STUDIES OF THE REDUCTION OF INORGANIC SULPHATE IN THE RUMEN OF SHEEP.

by

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Contents

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INTRODUCTION

The ability of ruminants to utilize inorganic sulphur was first indicated by Warth (1932) and has been confirmed by many workers in recent years (Moir *et al.*, 1967). The reduction of inorganic sulphate to sulphide has been demonstrated in the rumen (Lewis, 1954; Anderson, 1956) and *in vitro* (Henderickx, 1961) and inorganic sulphur amino acids of rumen microbial protein (Block *et al.*, 1951; Henderickx, 1961). Finally cysteine and methionine synthesized in the rumen have been isolated from tissue proteins of the host animal (Block and Stekol, 1950; Kulwich *et al.*, 1957).

Although a vast quantity of literature exists concerning the biology of micro-organisms that oxidize and reduce inorganic sulphur compounds, until recently little was known about the enzymatic mechanisms involved in the metabolism of these compounds. A large and diverse group of micro-organisms reduces sulphate in the small amounts required for the synthesis of cellular material, as evidenced by the ability of these organisms to grow on sulphate as their sole source of sulphur. This small-scale reduction of sulphate has been termed assimilatory sulphate reduction (Postgate, 1959). A much smaller group of micro-organisms reduces sulphate in great excess of nutritional requirements and produces massive amounts of sulphide. This large-scale reduction of sulphate to sulphide has been termed dissimilatory sulphate reduction (Postgate, 1959).

The chemical reduction of sulphate is a relatively difficult reaction and little progress was made on the mechanisms of biological sulphate reduction until Robbins and Lipmann (1958) simultaneously with Wilson and Bandurski (1958) elucidated the mechanism of sulphate activation in a number of organisms. Their observations, along with the initial observations of De Meio and Wizerkanisk (1956) are summarized in reviews by Gregory and Robbins (1960) and Wilson (1962). However, little if any published work relates to the mechanisms of sulphate reduction in the rumen.

This investigation was designed to show that the reduction of inorganic sulphate in the rumen of sheep followed a similar pathway as that of Robbins and Lipmann (1958) and Wilson and Bandurski (1958). The initial stages involved the isolation of the intermediates adenosine-5'-phosphosulphate (APS) and 3'-phospho -adenosine-5'-phosphosulphate (PAPS) from the rumen by use of S^{35}-labelled sodium sulphate and the remainder of the experimental work was designed to show by use of the group Vl anions - molybdate, selenate, chromate and tungstate - that the enzyme ATP-sulphurylase was involved in the activation of sulphate by ATP prior to reduction.

The review of literature covers the general sulphur metabolism and a further section discusses the bacterial sulphate reduction in more detail.

The experimental section of this thesis is divided into two parts. In part A, the isolation of APS and PAPS is presented. In section B the preliminary responses of the group Vl anions on inorganic

sulphate reduction are presented followed by a more detailed study of the responses to these group V1 anions. Finally the behaviour of pure species *Desulphovibrio desulphuricans* and sulphate-reducing bacteria isolated from the rumen in response to group V1 anions has been investigated.

Raw experimental data and statistical analyses have been recorded in the appendices.

REVIEW OF LITERATURE

1. Introduction

On current evidence there is no reason to believe that the tissues of ruminants differ in their qualitative nutrient requirements from those of other animals. However, domesticated ruminants which have been selected for a particular production function may differ greatly in their relative amino acid requirements from those of the feral ruminant. Also, the metabolic requirements of ruminants may be satisfied by diets that are quite inadequate for other mammals with "conventional" digestive tracts (Moir *et al.*, 1967-68). For example, ruminants may satisfy their tissue energy requirements from diets containing cellulose as the sole energy-yielding substrate, and their protein requirements from non-protein nitrogen and inorganic sulphur.

The special function associated with the reticulo-rumen is the digestion of cellulose and other carbohydrates by microbial activity. Rumen micro-organisms require a source of nitrogen, which is normally obtained from protein in the ingested feed, but may also be obtained from non-protein nitrogen in the feed, from urea secreted in the saliva, or from urea reaching the rumen by direct passage through the rumen wall (McDonald, 1948; Simmonet *et al.*, 1957).

Ruminants, through the medium of the reticulo-rumen and its symbiotic microbes are able to satisfy their metabolic requirements with dietary inorganic nitrogen and sulphur compounds and cellulosic compounds that are nutritionally inadequate for non-ruminant mammals (Garrigus, 1970).

The ruminant is a unique animal, since it is affected by the metabolism of the micro-organisms which exist in its rumen as well as by its own metabolism. In this symbiotic system the micro-organisms are provided a relatively constant environment for growth, and the end-products of their fermentation provide the ruminant with nutrients from feedstuffs which it would otherwise be unable to utilize. A number of factors, including type and amount of food consumed, the microbial population, and the animal itself, determine the balance of this complex ecological system and thereby affect the utilization of food by the animal.

The relationships between the components of the ruminant diet, the products of ruminal fermentation, and animal performance have received considerable attention (Barnett and Reid, 1961; Lewis, 1961; Allison *et al.*, 1964). Many of the micro-organisms which normally inhabit the rumen have been identified and characterized (Annison and Lewis, 1959; Bryant, 1959; Hungate, 1960, 1963) and their numbers have been determined under a number of dietary regimes (Warner, 1962). Several techniques, including use of total and differential counts (Bryant, 1959; Warner, 1962), washed cell-suspensions (Sijpesteijn and Elsden, 1952; Doetsch *et al.*, 1953), measurement of turnover rates and pool sizes of intermediates (Jayasurija and Hungate, 1959; Blackburn and Hungate, 1963) and the use of specifically labelled substrates (Baldwin *et al.*, 1963) have been successful in partially assessing the contributions of various microbial species and outlining metabolic pathways existent in the rumen mixed culture. However, as

yet the contributions of most ruminal micro-organisms to the total fermentation have not been satisfactorily measured. Since different bacterial species often possess different metabolic pathways, a study of these pathways in rumen bacterial extracts from animals fed different diets might be expected to reflect some of the differences in the bacterial population, and thus lead to a more comprehensive understanding of the contributions of both the individual combined micro-organisms with regard to rumen function.

The dietary concentration and quality of protein governs to a large extent the importance of the microbial phase of digestion. When the percentage of dietary protein is below about ten per cent the diet does not appear to meet the micro-organisms' protein requirements and it is considered (Hamilton *et al.*, 1948; Burroughs *et al.*, 1951) that in this situation 60-80 per cent of the protein digested by the animal in the abomasum is microbial protein which has been synthesized from non-protein nitrogen (Smuts *et al.*, 1941; Williams and Moir, 1951; Hungate, 1966; Purser and Buechler, 1966; Bergen *et al.*, 1967).

In conditions where the animal is receiving a low level of dietary protein it can be assumed that the micro-organisms will increase the amount of protein available to the animal by utilization of non-protein nitrogen and from the various forms of urea that are present. However, Chalmers (1961) considers that the quantity of protein synthesized in the rumen of sheep fed solely a urea diet may not be sufficient to support good growth, the degree of protein synthesis being limited by the availability of sulphur-containing amino acids.

Annison and Lewis (1959) found that with cows, after gastric digestion of microbial protein amino acids enter the circulatory system and tissue fluids and are subsequently used in the intermediary metabolism of tissue cells.

Hume *et al.* (1970) have shown that a close relationship exists between the level of intake of nitrogen (up to 9 g/day) and the amount of protein produced per day by the rumen micro-organisms. When the nitrogen intake was raised to 16 g/day, there was no further increase in the quantity synthesized in the rumen.

2. Animal Responses to Nitrogen and Sulphur

Several excellent reviews on nitrogen utilization by ruminants (McLaren, 1964; Phillipson, 1964; Blackburn, 1965; Hungate, 1966; Briggs, 1967; Waldo, 1968; Chalupa, 1968) have been written recently. In general these reviews have been concerned with results obtained mainly from feeding limited to moderate quantities of non-protein nitrogen (NPN) to ruminants.

The nitrogen potentially available to the host animal through digestion and absorption in the small intestine comprises undigested fodder protein and microbial protein (McDonald, 1952, 1954) together with small quantities of amino acids and peptides (Annison, 1956).

Sym (1938) was one of the first workers to realize that the proteolytic activity within the rumen was due to the micro-organisms

contained therein. This has since been substantiated by Pearson and Smith (1943), Warner (1956) and Blackburn and Hobson (1960). Most of the evidence that protein solubility is one of the factors influencing the rate of hydrolysis of protein within the rumen is indirect and relates to the high rumen ammonia concentrations found with a diet containing a soluble protein (Chalmers *et al.*, 1954; Chalmers and Synge, 1954; el-Shazly, 1952; Preston *et al.*, 1963). There is no extracellular, free proteolytic enzyme in the rumen, although all sheep rumen contents appear to have proteolytic activity irrespective of diet (Blackburn and Hobson, 1960), and thus there is a likelihood that any protein which enters the rumen will be hydrolyzed to some extent.

Microbial protein may constitute a major portion of the nitrogen-containing substances that arise in the lower digestive tract of the ruminant. Thus, Weller *et al.* (1958) observed that 80-90 per cent of omasal nitrogen content was microbial nitrogen, with ruminal bacteria accounting for 55-85 per cent of this nitrogen. The quality of this crude microbial protein has been investigated by a number of workers (Usuelli and Fiorini, 1938; Johnson *et al*, 1944; McNaught *et al.*, 1950, 1954; Clarke *et al.*, 1966; Hogan and Weston, 1967).

The large bacterial and protozoan fractions present in washed suspensions of rumen micro-organisms possess much of the total activity required to hydrolyze protein (Pearson and Smith, 1943; Warner, 1956; Hunt, 1957; Annison, 1956; Blackburn and Hobson, 1960). Thus, rumen bacteria must possess proteolytic activity, but initial

attempts at isolation of actively proteolytic strains resulted in the selection of facultative anaerobes and species which did not appear to be representative of predominant rumen bacteria (Appleby, 1955; Blackburn and Hobson, 1960).

There is an abundance of literature showing that ammonia is the end product of protein digestion within the rumen (Annison *et al.*, 1952; Annison, 1956; Warner, 1956; McDonald and Hall, 1957; el-Shazly, 1958; Lewis and McDonald, 1958; Annison and Lewis, 1959; Chalmers, 1961). These experiments show that the more soluble proteins give rise to larger concentrations of rumen ammonia. Proteolysis is a somewhat faster process than deamination (Annison, 1956; Blackburn and Hobson, 1960) and an increased concentration of amino acids and peptides may be evident immediately after feeding. Recently it was suggested (Purser and Buechler, 1966) that the amino acid compositions of acid hydrolysates of 22 individual strains of rumen bacteria were essentially similar to each other, and it was suggested that little variation in the protein quality of the bacterial fraction would occur as a result of variations in the composition of the population, unless differences in digestibility or amino acid availability between individual strains were important factors. A digestibility difference between species has been suggested by Pounden & Hibbs (1950). Thus, assessment of the protein quality of individual strains of rumen bacteria based solely on amino acid compositions or chemical scores may be in question.

Considering the fact that ammonia is a major nitrogen source within the rumen and that synthesis of microbial protein from ammonia

has been shown (McDonald, 1952), it is not surprising that many rumen bacteria utilize ammonia as a primary nitrogen source (Wasserman *et al.*, 1953; Gibbons and Doetsch, 1959; Phillipson *et al.*, 1959, 1962). It is clear that the predominant pattern of the nitrogen metabolism of pure cultures of rumen bacteria involves the synthesis of cell proteins from ammonia in preference to amino acids. Some recent work (Portugal, 1963) has confirmed that even within the rumen there is little incorporation of amino acids into bacterial protein. However, rumen bacteria have a marked capacity to de-aminate amino acids (el-Shazly, 1952); the ammonia produced may be utilized as a source of nitrogen by other micro-organisms, but large quantities may be absorbed into the blood stream and subsequently excreted as urea (McDonald, 1948). This reaction is potentially an important avenue of loss of ingested protein nitrogen. The extent to which such wasteful deamination occurs is related to dietary factors. Some types of protein give rise to much greater levels of rumen ammonia and urinary nitrogen loss than others (Chalmers and Synge, 1954); high levels of starch in the diet are favourable to maximum growth rates of rumen micro-organisms and to the utilization of ammonia, resulting in lower levels of rumen ammonia and urinary nitrogen (McDonald, 1952; Annison *et al.*, 1954). The increased efficiency of utilization of nitrogen by rumen microbes in animals on high starch diets may be the important mechanism involved in those experiments where wool growth responses were obtained following supplementation with starch (Ryder, 1968), but Ferguson (unpublished data) suggests that providing sheep are in energy balance at different

liveweights, wool growth was proportional to the digestible organic matter intake. Ferguson also stated that the non-protein portion of this intake has a relatively constant effect on wool growth, consistent with its effect on microbial synthesis in the rumen.

The ability of ruminants to utilize inorganic sulphur was first indicated by Warth (1932) and has been confirmed by many workers in recent years. Thomas *et al.* (1951) demonstrated conclusively that a sulphur deficiency may limit non-protein nitrogen utilization in purified diets and that sulphate-sulphur as a sole source of this element could correct the deficiency. Conversely, the use of non-protein nitrogen in ruminant rations increases the possibility of a sulphur deficiency (Johnson *et al.*, 1970) since the protein-rich feeds that have been replaced by urea are usually adequate sources of this element. Sulphur deficiency symptoms as far as is known are non specific and are confused with resultant low feed intakes. However, it has been shown that methionine, elemental sulphur or sulphate-sulphur supplementation of rations containing inadequate levels of sulphur have proved of benefit as judged by responses in terms of wool growth (Marston, 1935; Paducheva, 1961; Reis and Schinckel, 1963; Reis, 1965, 1968), body weight gain (Steyn, 1931, 1932; Thomas *et al.*, 1951) and nitrogen balance (Starks *et al.*, 1953). There is some controversy about the utilization of different chemical forms of sulphur by the ruminant animal. Albert *et al.* (1956) found that the chemical form of sulphur fed to young lambs affected dietary requirements. These workers found that the optimum sulphur level was about 0.17 per cent

sulphur when methionine was used to increase the S-content of a low -S basal diet against 0.32 per cent sulphur when sodium sulphate was used as the sulphur supplement and 0.40 per cent sulphur when elemental sulphur was used. Starks *et al.* (1953) showed that elemental sulphur added to a ration containing urea and formulated to be low in sulphur could be used by lambs to partially satisfy dietary needs for sulphur, and those lambs receiving sulphur retain more nitrogen than lambs receiving no additions of sulphur. On the other hand, Lofgreen *et al.* (1953) found that the addition of 0.2 per cent of sodium sulphate to a diet in which urea provided 40 per cent of the nitrogen was without effect on nitrogen retention and wool growth. However, Hale and Garrigus (1953) showed, with the use of labelled sulphur that sheep can synthesize cystine from elemental sulphur and sulphate, and that sulphate was more readily utilized than elemental sulphur.

Ammonium bisulphate has been found to be a good additive for ensilage of grass or grass-clover mixtures when added at a level of about 16 lb per ton of fresh herbage (McCarrick, 1962, 1964). However, voluntary intake by cattle of ammonium bisulphate silage is much less than that of similar silage preserved with molasses (McCarrick *et al.*, 1965a), the reduction in intake being directly proportional to the amount of salt added (McCarrick *et al*, 1965b). Two suggestions have been put forward to explain the reduced voluntary intake. One is that ammonium bisulphate disturbs the acid-base equilibrium of the animals due to its acid bisulphate radical (McCarrick *et al*, 1965c). These authors observed that the salt induced metabolic acidosis in cattle as

indicated by plasma CO_2 and urine pH values. L'Estrange *et al.* (1969) fed sheep a supplement of either sodium sulphate, sodium bisulphate, ammonium bisulphate or ammonium sulphate, each in amounts equivalent to one per cent sulphur on a dry matter basis. Sodium sulphate and sodium bisulphate decreased voluntary intake by 22 per cent and each of the other supplements decreased it by 44 per cent. Reduction in intake was related to the degree of metabolic acidosis induced by the respective supplements, except for sodium sulphate. Feed intake returned to normal immediately after withdrawal of each supplement.

Many workers have suggested various indices for measuring the sulphur status of plants. Dijkshoorn *et al.* (1960) proposed that where the nitrate N content of the plant was low relative to the total N, the organic S:organic N ratio (expressed in g atoms/Kg D.M.) would approximate 0.027. This is equivalent to an N:S ratio of 16.2. Later Dijkshoorn and Van Wijk (1967) suggested an organic S:organic N ratio of 0.032 for gramineous plants. Odelein (1963) reported an experiment in which the N:S ratio in oats straw was 6.0 where sulphur was adequate, and 49.3 where sulphur was deficient. The wide variation in N:S ratio suggests that this index may prove useful for estimating the S status of plant material. Jones and Mortimer (1964) suggested that the sulphate content of rose and subterranean clovers, sampled during flowering and before wilting, provided a good estimate of the sulphur status of soil. Blair and Crofts (1970) found that sulphur fertilization increased the dry matter yield of autumn sown, high density oats adequately supplied with nitrogen fertilizer, grown on previously unfertilized basaltic

and granitic soils of the Central Tablelands of New South Wales. Chemical analyses indicated that, under the conditions of these experiments, sulphur was insufficient for plant growth when the immature oats herbage had a sulphate S content less than 0.04 per cent, a total S content of less than 0.10 per cent and an N:S ratio in the range of 20 to 50.

The total sulphur content of a plant changes more than does its sulphur-amino acid content with changes in the level of available sulphur in the soil (Allaway and Thompson, 1966). Sulphur-adequate plants generally contain more sulphur-amino acids and are presumably of better nutritional quality for animals than are sulphur-deficient plants. Generally, diets deficient in the sulphur-containing amino acids have caused a reduction in voluntary food consumption by other animal species (Kumata and Harper, 1962; Sanahuja *et al.*, 1965). Since sulphur is an integral part of the methionine and cyst(e)ine molecule then it would be expected that inadequate levels of sulphur in the diet would limit the synthesis of these sulphur amino acids in the rumen and thus in turn limit the synthesis of microbial protein.

Sulphates are present in most water supplies because of their solubility as ground sulphates. According to the drinking water standards of the U.S. Public Health Service (1962) sulphate should not be present in a drinking water supply in excess of 250 mg/litre if more suitable supplies are or can be made available. There is information on the tolerance of animals (Heller and Paul, 1934; Heller and Haddad, 1936; Ballantyne, 1957; Pierce, 1960) including cattle (Embry *et al.*, 1959)

for sulphate in the drinking water. From a review of information available, McKee and Wolf (1963) assumed that water containing 500 mg/litre of sulphate would not be detrimental to livestock. Bray (1964) indicated that sheep drinking water with a high content of sulphate possibly could consume up to 1.5 g of sulphur per day. However, the data of Altman and Dittmer (1964, 1968 - cited by Garrigus, 1970) makes Bray's estimates appear conservative, as these authors cite instances of sulphate ground water having 2327 mg/litre of sulphate, and these authors stated that sulphur as sulphate may constitute 70.5 per cent of the total solids in sulphate ground water.

Apart from some minor exceptions sulphide is probably the central metabolite in rumen sulphide metabolism (Moir, 1970). Moir also stated that the utilization of dietary sulphur depends not only upon the quantity and nature of available sulphur, but upon (1) the rate of sulphide production, (2) the uptake of sulphide by the microorganisms, and (3) the loss of sulphide from the rumen. The rate of sulphide production is rapid (Lewis, 1954; Anderson, 1956; Halverson *et al.*, 1968; Spais *et al.*, 1968; Bray, 1969a), whether it is from sulphate, more reduced inorganic substrates and cysteine or cystine. Bray (1969a) added carrier-free S^{35} sulphate per rumen and the maximum blood sulphur -35 was reached within two hours after administration. The concentration of sulphide in the rumen liquor in this same experiment rose to a peak within an hour of feeding and then after a period of eight hours, gradually declined to pre-feeding levels by next morning. Radioactivity could only be demonstrated in the rumen

sulphide fraction in the first hour after administration of S^{35}-sulphate per rumen.

The sulphide produced in the rumen can alternatively be absorbed across the rumen wall (Anderson, 1956; Bray, 1969b), incorporated into sulphur amino acids or lost by passage down the gastrointestinal tract (Block *et al.*, 1951; Henderickx, 1961). It has been shown (Bird and Hume, 1971) that the form of dietary S supplement may influence flow characteristics of sulphur from the rumen. When sheep were fed supplements of sulphate-sulphur, cystine sulphur or sulphate-sulphur plus cystine, only the cystine treatment increased the flow of sulphide, ester sulphate, soluble organic sulphur and cystine to the omasum, and increased the ruminal sulphide concentrations above basal.

Although Anderson (1956) used very high levels of sulphide, he did not find any toxic symptoms, even when he added up to 113 µg per ml. In Bray's (1969b) experiment, however, when sulphide levels up to 330 µg per ml were added to the rumen in which normal rumen contents were replaced with three litres of buffer solution, sulphide could be detected on the animals' breath in 10-15 minutes. The concentration of sulphide in the rumen of Bray's sheep decreased in a log linear fashion with time and the half-life of the rumen sulphide obtained in the three experiments reported were 21.5, 15.5 and 10.7 minutes prespectively. Bray calculated a half-life value of 17 minutes from Anderson's (1956) data and Moir (1970) estimated a value of 35 minutes from the data of Spais *et al.*, (1968). Anderson's data is logarithmically linear over the first 30 minutes, but after 30 minutes

the logarithmic concentration plot against time is non-linear. Bray (1969b) has postulated that the findings of Anderson (1956) suggest that absorption rates of sulphide from the normal rumen are similar to those observed in Bray's washed rumen experiments.

In normal animals the sulphur of degraded methionine and cystine is excreted mainly as inorganic sulphate, although α-keto-γ-methylthiobutyric acid (the keto acid derived from methionine) and methionine sulphoxide have also been detected as urinary constituents. The excretion of sulphur in the urine closely parallels the nitrogen excretion. This was adequately demonstrated by Sherman and Hawke (1900). Wright *et al.* (1960) reported a correlation between urinary nitrogen and sulphur excretion in pre-adolescent children at low levels of dietary protein, but not at high levels. However, in dogs Bressani *et al.* (1965) found excretion in the urine of these two elements to be parallel. Since by far the largest store of sulphur in the body is in the form of cystine and methionine in the proteins, the sulphur turnover in the body should reflect the protein turnover. Friedberg *et al.* (1948), Tarver and Morse (1948) and Solomon and Tarver (1952) used single doses of S^{35}-methionine to estimate protein turnover in liver, kidney, plasma and muscle in rats by measuring the change in specific activity of the methionine sulphur in a four day period. If the proteins in the whole body were thoroughly and uniformly labelled with S^{35}, the excretion of this label in the urine would be proportional to the protein catabolic rate. Jackson (1968) labelled the body proteins of rats with S^{35} by the daily administration of S^{35}-L-methionine for a four week

loading period. From the pattern of S^{35} excretion it was proposed that the source of S^{35} was two metabolic pools, one with a turnover rate of about seven per cent per day (half-life of 10 days) and the other with a turnover of about 0.4 per cent per day (half-life of 175 days).

In an experiment using S^{35} labelled elemental sulphur, sodium sulphate and methionine given per OS in lambs, Johnson *et al.* (1970) found different excretion patterns of S^{35} for each of the three chemical forms in the subsequent eight day collection period. The peak urinary excretion of S^{35} from dietary sulphate-sulphur occurred during the first 0.25 days after dosing.

In the experiments of Johnson *et al.* (1970) the greatest amount of S^{35} from dietary methionine was found in the urine 1-1.5 days after dosing while the greatest amount of radioactivity from S^{35} elemental sulphur was found in the urine between 1.25 and 1.5 days after dosing. These workers found the major pathway of excretion of S^{35} from elemental sulphur and methionine was via the faeces. Losses of S^{35} from sodium sulphate were about equally divided between faeces and urine.

Bray and Hemsley (1969) found an increase in faecal sulphur per 100 g dry matter intake with increasing dietary sulphur levels, and the faecal nitrogen to faecal sulphur ratio decreased with increasing levels of dietary sulphur. Barrow and Lambourne (1962) studying sheep fed on a pasture material without sulphur supplements, found that dietary sulphur levels did not affect faecal sulphur excretion, and that an average of 0.114 g sulphur was excreted in the

faeces per 100 g of dry matter eaten. These authors suggested that the variation between their results and those of other workers may be due to differences of sulphur digestibility between feeds, which would be an important factor in considering minimum sulphur requirements. Bird and Thornton (unpublished data), however, found increased faecal excretion of sulphur by up to 110 mg per day and of S^{35} following intravenous infusion of $Na_2S^{35}O_4$ to sheep fitted with a re-entrant ileal cannula, and decreased urinary excretion of sulphur and of S^{35} by similar amounts after feeding sheep an average of 9.5 g N per day and 1.30 g S per day. Four levels of infused glucose were imposed on this diet at the rate of 0, 30, 60 or 90 g per day into the distal ileum. These infusions altered the sulphur output in the faeces and urine without affecting the retention of sulphur or of S^{35}. Increases in the faecal output of organic sulphur and S^{35} were due mainly to an increased output of organic sulphur and S^{35}, while the decrease in urinary sulphur excretion was due largely to a decreased output of inorganic sulphate.

Bird (1971) found that infusion of sulphate either intraruminally or intraduodenally increased the excretion of total sulphur in faeces and the excretion of total sulphur, ester sulphate and inorganic sulphate in the urine. Inorganic sulphate excretion in faeces was increased significantly only by intraduodenal infusions, and the excretion of neutral sulphur in faeces and urine only by intraruminal infusions. It would appear that the site of infusion and the substrate being infused is the important factor in increasing or decreasing the out put of sulphur.

Bird (1971) further suggested that the intake of sulphur and the supply of digestible energy to the fermentative rumen and hindgut regions primarily determined the amount of organic sulphur excreted in the faeces by affecting the synthesis of bacterial sulphur. These results indicate that the ruminants ability to metabolize ingested sulphate is substantially influenced by its ruminal micro-organisms.

Balance studies in which fractionation of sulphur between urine and faeces is used as an index of sulphur absorption from the gut must be treated with caution in interpreting sulphate absorption data. In a series of experiments involving continuous infusions of sulphate into the duodenum of sheep, Moir, Somers and Bray (1967-68) reported the appearance of hydrogen sulphide in the faeces, which they took as presumptive evidence of sulphate reduction in the large intestine. Bray's (1969a) experiments showed that the rapid excretion of S^{35} in the urine and the pattern of the urinary total sulphur excretion followed a similar pattern to that of renal regulation which limits blood inorganic sulphate (McLean, 1960). Bray also found that after dosing with S^{35}-sulphate intravenously in two separate experiments, 19 and 12 per cent of the dose was recovered in the faeces. The bulk of this must have been due to sulphate secretion into the post-ruminal alimentary tract, since rumen and saliva S^{35} levels at their maxima would have accounted for less than one per cent of the dose.

Bird and Moir (1971) found that the mean absorptive capacity of the entire intestine when sodium sulphate was continuously infused into the rumen or duodenum of four sheep in amounts of 0, 1.5, 3.0 and

6.0 g per day, was up to 5 g of sulphate sulphur daily. These workers also estimated the rate of uptake of sulphur from duodenally infused sodium sulphate into the blood to be of the order of 110-150 mg per hour and up to 3.5 g sulphate sulphur could have been absorbed daily from the small intestine. This occurs even though the sulphate ion is considered to be poorly absorbed from the intestine of humans and dogs (Kun, 1961). The possibility exists (Bird and Moir, 1971) that at least part of the sulphate was reduced by the intestinal bacteria to sulphide, since under certain conditions the small intestine of many animals may host large numbers of anaerobic bacteria (Donaldson, 1968).

The fact that sulphide is more readily absorbed than sulphate following the introduction of S^{35}-sulphide per duodenum (Bray, 1969a) indicated that the sulphate ion is poorly absorbed (Kun, 1961) especially when compared with cysteine or chloride ion (Andrews and Johnson, 1933; Ingraham and Vischer, 1936). The results of Bray (1969a) help to substantiate this claim, when a virtual absence of S^{35} in the faeces of sheep given S^{35}-sulphide per duodenum against the recovery of 35-41 per cent of the dose in the faeces of sheep given S^{35} sulphate per duodenum was obtained. He also found a more rapid excretion of S^{35} derived from sulphide than of S^{35} derived from sulphate in the urine. On present evidence it may be stated that sulphur not in organic form is lost largely by absorption of sulphide directly through the rumen wall (Moir, 1970).

The data in Table 1, taken from Moir (1970), illustrates work done by Bird and Hume (1969) on the flow pattern of sulphur products from the rumen through the omasum.

Table 1

Rumen Sulphur Data

	Treatments			
	A basal	B +SO_4	C +cystine	D +SO_4,+cystine
S-intake (mg)	610	1940	1950	3420
S-outflow total (mg)	1072	1227	1260	1505
Inorg. SO_4-S outflow (mg)	28	65	49	81
Sulphide-S flow (mg)	4	21	37	44
Sulphur gain or loss (mg)	+460	-713	-690	-1915

The net daily addition to the ruminal digesta on the basal diet was 460 mg sulphur above that ingested. This level is much greater than the values given by Bray (1964) who estimated the return of sulphur to the rumen via saliva to be 138 and 180 mg per day, based on the sulphur content of saliva collected, and estimated flow rates. Bird and Hume (1971) estimated a net daily addition of 446 mg sulphur by measuring sulphur flow at the omasum and by difference with dietary intake. Bray (1969c) injected S^{35} labelled sodium sulphate intravenously into three sheep in which the normal rumen digesta had been replaced with buffer solutions, hypotonic, isotonic and hypertonic to blood showed that inorganic S^{35} sulphate was transferred to the rumen by passage across the rumen wall and that the rate of passage of sulphate was apparently increased by the influx of water into the rumen, however quantitative transfer was small.

3. The Effects of Molybdenum in the Diet

The first observations on the role of molybdenum in animal nutrition were concerned with the toxic effects of this element. Ferguson *et al.* (1938) reported that in certain restricted areas in Great Britain a cattle disease, known locally as "teart", and characterized by extremely severe diarrhoea and loss of condition was attributable to the high molybdenum content of the pastures on which the animals grazed. These investigators were able to produce a similar condition in cattle either by dosing the animals with a molybdenum salt, or by raising the molybdenum content of a pasture by application of molybdenum fertilizers. Similar toxicities in cattle grazing on pastures of high molybdenum content (15 to 300 ppm dry weight) have since been reported from America (Barshad, 1948), Canada (Cunningham *et al.*, 1953), New Zealand (Cunningham, 1950) and Sweden (Hallgren *et al.*, 1954).

Dietary intakes of copper, molybdenum and sulphate form a most complex set of interactions with profound effects on the animal, but the mechanisms involved are still obscure. In the non-ruminant animal supplements of molybdate salts depress growth and may restrict haemoglobin production (Dick, 1956a). Several workers (Comar *et al.*, 1949; Miller *et al.* 1956a) have observed that molybdenum exerts its adverse effects on copper utilization in the rat, not by preventing copper absorption, but by apparently restricting its mobilization from tissue stores. Examples of this have been found in rats receiving molybdenum supplements in

which their liver copper stores may be abnormally high, and yet supplementary copper improves growth.

In ruminants a high molybdenum intake can induce a copper deficiency even when the copper content of the pasture is quite high; the effect can be prevented by providing an increased copper intake. Two separate mechanisms may be involved here, that of the provision of copper to the tissues, and secondly the way in which copper prevents toxic reactions due to molybdenum itself. Excess dietary molybdenum limits the retention of copper in the body (Dick, 1954), an activity that is dependent on a sufficiently high sulphate or sulphur intake. Similarly, high intakes of copper or sulphate tend to reduce the retention of molybdenum (Dick, 1956). In this work Dick found that the limiting effect on copper storage by molybdenum, within the range 2.5-25.0 mg/day progressively increased as the inorganic sulphate was increased between 1 and 6 g per day. Mylrea (1958) also found that molybdenum and sulphate can influence the copper status of cattle. Increasing the molybdenum from 2.4 ppm to 9.2 ppm caused the liver copper concentrations to drop from 144 ppm to 63 ppm in steers receiving 0.55 per cent sulphate ration, but had no effect on steers on a 0.03 per cent sulphate ration. While it is probably sulphate in the tissues that interacts with molybdenum, any dietary S that promotes sulphate production in tissues will have a similar effect.

Cunningham (1950) and Cunningham *et al.* (1956) reported levels of 7 ppm copper and more than 5 ppm molybdenum for pastures in New Zealand where "complicated" copper deficiency occurs. While these

workers did not publish any figures on sulphate levels it would seem, in view of the results of Mylrea (1958) that a moderate sulphate content would be sufficient to cause low liver copper levels.

Although it has been reported that the molybdenum ingested is readily absorbed from the intestinal tract and excreted largely in the urine (Comar, 1950; Ferguson *et al.*, 1938), studies in sheep have shown that both the amount absorbed by the animal and the route of excretion of the absorbed molybdenum depend on the amount of inorganic sulphate in the diet (Dick, 1953, 1955). In these experiments it was shown that the molybdenum concentration in the blood of the animal reflected the intake when the inorganic sulphate was dependent upon the amount of sulphate in the diet. For example, when the daily intake of sulphate was 1.8 g per day and the daily intake of molybdenum was 0.4 mg per day, the blood molybdenum level was 2 µg/100 ml, while at the same sulphate intake and 96 mg molybdenum, the blood molybdenum level rose to 495 µg/100 ml. Sheep given a constant daily intake of 15 mg molybdenum and fed 1.1 g sulphate had a blood molybdenum level of 114 µg/100 ml whereas when the sulphate intake was increased to 5.7 g per day the blood molybdenum level dropped to 29 µg/100 ml. In the latter case the excretion of molybdenum in the urine rose rapidly and led to the fall in the blood-molybdenum concentration. The amount of molybdenum excreted in the faeces was also found to be increased by administration of sulphate, which indicated a reduction in the rate of molybdenum absorption from the gut. If the same molybdenum intake was continued, but no further sulphate given, within a few days the urinary excretion

of molybdenum fell and the blood-molybdenum level rose again to the pre-experimental figures.

On the other hand, if sulphate administration was continued as a daily dose, the blood-molybdenum level continued to fall until it reached a steady value dependent on the sulphate intake, but the amount of molybdenum excreted in the urine will constitute a lower proportion of the intake than it did before the administration of the sulphate. These effects of inorganic sulphate in reducing molybdenum absorption and increasing the loss of stored molybdenum were reflected in a lower tissue content of molybdenum.

Rapid absorption of molybdenum from the intestinal tract has been reported by Ferguson *et al.* (1938) using non-radioactive molybdenum, and Comar *et al.* (1949) and Shirley *et al.* (1954) using radioactive molybdenum. The route of excretion of molybdenum is mainly in the urine in the rat and sheep (Miller *et al.*, 1956). The sulphate effect is highly specific and, in sheep, is not shared by anions such as tungstate, selenate, silicate, malonate and citrate (Dick, 1956; Scaife, 1956). The influence of sulphate on molybdenum absorption and excretion is explained by Dick (1956) on the hypothesis that inorganic sulphate interferes with, and if the concentration is high enough, prevents the transport of molybdenum across membranes.

Studies by Marcilese *et al.* (1970) indicated that the addition of molybdenum plus inorganic sulphate to the diet resulted in greater accumulation of copper in the kidney and its increased excretion by way of the urine. This work confirmed other reports in both sheep

(Smith *et al.*, 1968) and rabbits (Gaballah *et al.*, 1965) in which there was an increase in the concentration of intravenously injected Cu^{64} in the kidney with dietary supplements of molybdenum and sulphate.

It is apparent that dietary supplements containing inorganic sulphate promote the urinary excretion of molybdenum in the rat and the sheep and restrict or completely prevent its accumulation in the liver (Miller *et al.*, 1956). The effect of sulphate in preventing molybdenum intoxication is shared by many inorganic sulphur compounds that are oxidized to sulphate during metabolism, as has been shown by Van Reen and Williams (1956), Dick (1956) and Miller and Denton (1959). In contrast, the presence of sulphides in the diet has been found to aggravate molybdenum toxicity in the rat (Mills, 1960). Further studies of this point have led to the finding that molybdenum intoxication restricts the oxidation of sulphide in liver tissue (Mills *et al.*, 1958). This result explains why organic sulphur compounds, e.g. methionine, that are normally degraded by a pathway involving endogenous release of sulphide and its subsequent oxidation to sulphate, fail to protect against molybdenum intoxication if molybdenum has already accumulated in the tissues (Halverson *et al.*, 1961).

4. Rumen Metabolism

Normally there exists a considerable variation in rumen pH on a wide variety of diets. This variation is usually positively associated with fermentation activity. Quoted values range from 5.0 to 7.5, but it is unlikely that cellulose digestion takes place efficiently at a pH much lower than 6.5 (Phillipson *et al* 1962) due to the reduction in microbial protein synthesis.

The pH of rumen contents varies with time (Monroe and Perkins, 1939; Briggs *et al*, 1957; Brethour *et al*., 1958). The greater proportion of this variation is due to volatile fatty acid formation (Monroe and Perkins, 1939; Phillipson, 1942; Chance *et al*., 1953). The pH drops after feeding and falls to its lowest level some six hours later. Thereafter it rises slowly to its initial level.

The rates of production of volatile fatty acids vary (Stewart *et al*, 1958; Hungate *et al*., 1961) since the amount of any substrate in the rumen varies with time due to the variation in consumption and fermentation. It has been found that the end-products of fermentation of most substrates by most rumen micro-organisms consist largely of volatile fatty acids (Heald and Oxford, 1953; Bryant, 1959; Hungate, 1960; Abou Akkada and Howard, 1960; Williams *et al*., 1961). Warner (1964) tabulated data on the rates of production of volatile fatty acids in the rumen of sheep, and estimated that a sheep of 35 Kg body weight would produce one mole of total VFA per day or 74 m mole per $Kg^{0.73}$, while a sheep of 27 Kg body weight would produce 1.75 mole

total VFA or 157 m mole per $Kg^{0.73}$ per day. Bergman *et al.* (1965) determined the "gross" rate of production of each volatile fatty acid by infusion of the corresponding labelled acids one at a time into the rumen of a sheep fed continuously throughout 24 hours. These authors were able to determine from their single acid infusions into sheep fed on grass hay, the net amount of butyric acid which had been derived from acetic acid by interconversion, and concluded that 51-66 per cent of it originated in this way. Experiments of Bergman *et al.* (1965) and Weller *et al.* (1967) show that the mixture of acids finally produced in the rumen has a composition very similar to that of the mixture present in the rumen fluid.

The rate of entry of saliva into the rumen (Somers, 1957; Balch, 1958; Kay, 1960; Bailey, 1961) and the rate of outflow of material from the rumen (Murray *et al.*, 1962) vary with time. Water can cross the rumen wall in response to change in osmotic pressure after eating, drinking or release of anions (Danielli *et al.*, 1945). The total amount of any fatty acid in the rumen thus varies considerably with time. Blaxter (1964) has stated that rumen pH is maintained within a narrow range by the continuous influx of food and water and by a continuous inflow of buffered saliva.

The fatty acids are absorbed into the blood, mainly through the rumen wall (Phillipson, 1947; Gray, 1947; Masson and Phillipson, 1951; Annison and Pennington, 1954). Other energy-yielding substances are not absorbed in amounts sufficient to supply any large part of the energy needs of the animal. Little glucose passes out of the abomasum

(Heald, 1951) or is absorbed into the blood (Schambye, 1951; Annison *et al* ., 1957; Fries and Conner, 1960) and there is some evidence that adult ruminants may not be able to absorb large amounts of glucose from the gastro-intestinal tract (Huber *et al.*, 1961).

Studies of absorption of VFA from the rumen, conducted by adding solutions of VFA to the washed-out rumen, have shown that the rate of absorption is directly related to the VFA concentration and chain length (Masson and Phillipson, 1951).

The requirements of the cellulose-digesting bacteria for the carbon skeletons of the higher VFA for growth are now established (Bryant and Doetsch, 1955; Allison *et al.*, 1958, 1962). Since these fatty acids arise from the breakdown of amino acids (el-Shazly, 1952) it may be postulated that the addition of these growth factors to protein-free diets should result in an increase in the amount of microbial protein produced in the rumen. Some of the undegraded protein, in addition to the microbial protein, passes to the abomasum when the solubility of the dietary protein is low. Thus, as well as absorption of VFA across the rumen wall, some of the loss of VFA from the rumen must be due to passage down the intestinal tract.

While several studies have been devoted to determining the influence of various factors on the pH of rumen contents, relatively few have been reported on the redox potential, which may serve as a criterion of microbiological activity in the rumen. The work of Broberg (1957, 1958) in this field is therefore of considerable interest.

In a series of studies carried out under both *in vitro* and *in vivo* conditions he found that the rumen contents of healthy animals had a well-defined potential of about -400 mV when measured with a platinum electrode. However, Broberg (1957) stated that the use of platinum electrodes was associated with an experimental error of ± 20 mV. Sheep fed a normal diet had rH values of 8.3 ± 0.6, and feeding straw alone or large amounts of protein did not alter this value. When concentrates were fed, a temporary drop in the rH value to 6.9 ± 0.09 occurred whereas acute over-eating of grain led to a large increase in the rH value. Similarly, high values of the order of 11.2 ± 0.76 were obtained in cases of anorexia or depressed appetite over an extended period which may in turn have led to conditions of starvation. Some cases of bloat also led to such high values. However, the redox potential in sick animals was shown to be stable. By further experimentation involving the measurement of redox potential and gas analyses, Broberg demonstrated the very high capacity of rumen ingesta to utilize atmospheric oxygen. By passing oxygen through the rumen contents of a sheep very rapidly the rH level within the rumen increased, but no effect could be obtained when a slow stream of gas was bubbled through for three days. Nor did rumination have any apparent influence on the rH level. Baldwin and Emery (1959) confirmed that the addition of controlled quantities of oxygen to rumen contents incubated *in vitro* was without effect on the redox potential or the production of gas, volatile fatty acid or ammonia in the fermentation medium.

In rumen microbiological studies it is important to firstly classify the ruminal micro-organisms into two groups - ciliate protozoa and the bacteria. Reactions in the rumen are, in the main, the results of symbiotic relationships between both the protozoa and the bacteria, and also between the protozoa and the bacteria individually. Gall and Huhtanen (1951) suggested that before an organism can be looked upon as a typical component of the micro-floral population the following main criteria should be established:

(a) the organism must be able to live anaerobically;

(b) it should be able to produce the type of end-product found in the rumen;

(c) the rumen ingesta should contain not less than one million of the particular organism per gram of rumen digesta.

Some workers such as Baker (1943) and Moir and Masson (1952) have distinguished bacteria on morphological characters, while others have cultured these bacteria. Two factors influencing the numbers and kinds of micro-organisms within the rumen have been widely recognized as important; the nature of the ration and the time interval since feeding. Mowry and Becker (1930) noted a considerable increase in the numbers of protozoa on adding starch and protein to a hay diet, while the effects of different kinds and amounts of protein, with or without starch, on the total bacterial count have been investigated by Moir and co-workers (Moir and Williams, 1950; Williams and Moir, 1951; Williams *et al.*, 1953). Qualitative differences in the rumen bacterial

population with different rations have been found by Gall *et al.* (1949), Burroughs *et al.* (1950), Spisni (1955) and Maki and Foster (1957).

Hungate (1947) has succeeded in isolating cellulose-digesting bacteria from high dilutions of rumen contents. Van der Wath *et al.* (1948) have grown several rumen bacteria, one being an iodophilic coccus isolated from sheep, and Van der Wath [redacted]. (1948) have also made direct counts of rumen micro-organisms finding an average of 1-2 x 10^9 per ml of rumen contents. Kohler (1940) studied numbers of rumen bacteria by several techniques. By direct count he found approximately 1.3 x 10^{10} per gram while Gall *et al.* (1949) using a similar technique measured numbers ranging from 5-10 x 10^{10} per gram of fresh rumen contents. This direct count does not measure the total viable bacteria numbers since all bacteria either dead or living are included. Both Kohler (1940) and Van der Wath *et al.* (1948) stated in their publications that their direct counts were too low. If viability (defined by Mara and Williams, 1970, as the ratio of colony count to the total microscopical count) is to be taken as the sole criterion for evaluating enumerative media and methods, it would seem desirable to use only cultures known to be growing exponentially, even though bacteria in this growth phase are often hypersensitive to stresses such as dilution shock and changes in temperature and the Eh value of their environment.

Warner (1962) defined the standard count as the concentration of micro-organisms found in strained rumen liquor, and the total count as the concentration found in whole rumen contents after the feed

particles had been washed and disintegrated. The concentration of the organisms as measured by the standard count can be affected by dilution of the rumen liquor, by division or death and lysis of organisms, by engulfment of other organisms, and by sequestration of organisms in and return from places not sampled at examination, such as close to the rumen wall, or in close contact with the solid mass of feed found with some diets.

Diurnal fluctuation of microbial numbers, related to the time after feeding, is commonly recognized. Warner (1966a) found that the pattern of change of concentration of different groups of micro-organisms in the rumen of sheep fed limited diets once daily was found to be characteristic of the group and little affected by the time of day, the nature of the diet or the host animal. When sheep were fed a limited ration every three hours (Warner, 1966b) a three hour rhythm of concentration change was seen for most micro-organisms. Superimposed on this was a gradual decline in concentration of several groups of micro-organisms, presumably due to the removal of nutrients by repeated sampling from the rumen. However, in natural circumstances (Warner, 1966c) most ruminants have virtually unlimited amounts of feed available and may choose their own times and rates of eating. Ruminants at pasture usually graze for 8-9 hours per day, and most of this time is spent in one major period of fairly continuous eating (Hughes and Reid, 1951; Arnold, 1962). The rumens of sheep fed in this way and also in those fed once daily (Warner, 1966a) were more tightly packed than in those fed a limited ration every three hours (Warner,

1966b), and hence the digesta were presumably less well mixed. Thus it appears that the conditions which control microbial growth in the rumens of animals feeding naturally to appetite, whether in pens or at pasture, resemble those in animals fed a restricted diet once daily, presumably owing to the basic similarity of eating behaviour.

The sulphate-reducing bacteria form a specialized group of microbes that use sulphate as a terminal electron acceptor for their respiration. Though many microbes generate sulphide metabolically, sulphate often being the primary source of that H_2S, the process is normally a small scale one involving the incorporation of sulphur into cell protein and its subsequent degradation by catabolic and autolytic processes. These sulphate-reducing bacteria have been distinguished, as either assimilatory or dissimilatory sulphate reducers (Postgate, 1959). Yeasts carry out assimilatory sulphate reduction, as evidenced by their ability to grow on sulphate as their sole source of sulphur (Schultz and McManus, 1949). Cell-free extracts of yeast have been prepared that will reduce sulphate to sulphite and also sulphide (Wilson and Bandurski, 1958; Hilz *et al.*, 1959). This reduction requires, in addition to enzyme and sulphate, adenosine triphosphate (ATP) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) or reduced lipoic acid as electron donor (Hilz and Kittler, 1958). The dissimilatory group of sulphate-reducing bacteria use sulphate as an electron acceptor and release H_2S. Surprisingly few bona fide dissimilatory sulphate-reducers have been described, and a large portion of the physiological and enzymological observations concerning the reduction

of sulphate have been made with the classic sulphate reducer, *Desulphovibrio desulphuricans*. The original type species *D. sulphuricans (Spirillum desulphuricans)* was described by Biejerinck (1895) and isolated in pure culture by van Delden (1903). A comprehensive study of its physiology and metabolism was made by Baars (1930). The dissimilatory sulphate-reducing bacteria are widely distributed in sea water, marine muds, fresh water, soil and oil-bearing environments.

Campbell and Postgate (1965) and Postgate and Campbell (1966) distinguished two genera; *Desulphovibrio* (Kluyver and van Neil, 1936); mesophilic, non-sporulating, sulphate-reducing bacteria and *Desulphotomaculum* (formerly *Clostridium*); spore-forming bacteria with both mesophilic and thermophilic representatives.

The current classification is supported to some extent by the infrared spectral properties of sulphate-reducing bacteria (Booth *et al.*, 1966). The thermophilic bacterium, *Desulphotomaculum nigrificans*, formerly known as *Clostridium nigrificans*, is clearly distinguished from species of *Clostridium* on the basis of its DNA-base composition (Saunders *et al.*, 1964; Saunders and Campbell, 1966). The genus *Desulphovibrio* appears to consist of three main groups on the basis of its DNA-base composition (Sigah *et al.*, 1963; Saunders *et al.*, 1964; Miller *et al.*, 1968) and on tolerance to bis-p-chlorophenyldiguanidine-hexane diacetate (Saleh, 1964).

All dissimilatory sulphate-reducing bacteria are strict anaerobes and, in general, media must be poised at a low Eh (0 to -100 mV) for rapid growth to occur (Grossman and Postgate, 1953;

Abd-el-Malek and Rizk, [redacted], 1960; Postgate, 1959a; Alico and Liegey, 1966). *Desulphovibrio* cultures normally show linear growth and provide excellent examples of such linear growth, typical growth curves having been provided by Senez (1951). Linear growth may arise for three reasons: (1) sulphide accumulated in the cultures during growth and acts as a growth inhibitor, (2) sulphide precipitates iron, which is required as a micro-nutrient and thus decreases its availability; (3) sulphide evaporates during growth, thus lowering the pH value of the culture. This pH change alters both growth rate and yield and, in extreme cases, can cause alkaline lysis of the population (Postgate, 1965).

Postgate (1959, 1965) and Postgate and Campbell (1966) reviewed procedures for the enrichment, isolation and mass culture of these bacteria and a technique for the continuous culture of *Desulphovibrio* has been reported by Leban *et al.* (1966), and an apparatus for the continuous cultivation of sulphate-reducing bacteria in general has been reported by Hallberg (1970). It is often necessary to obtain a reliable estimation of the viable numbers of these sulphate-reducing bacteria in either a natural environment or a laboratory culture. Media based on the lactate-sulphate prescription of Baars (1930) are used for enumeration of *Desulphovibrio* species. Later workers have improved the original Baar's medium, and Butlin *et al.* (1949) showed that the incorporation of 0.1 per cent (w/v) of yeast extract enhanced growth, and Grossman and Postgate (1953) recommended the use of reducing agents, especially in enumerative work. The formation of black colonies due to

the reduction of the ferrous sulphate to ferrous sulphide facilitates the determination of viable count. Counting procedures have been restricted to the most probable number multiple tube technique (Hata *et al.*, 1964; Pankhurst, 1968), colony counts in deep agar "shake" tubes (Grossman and Postgate, 1953) and the rolltube technique (Toerien *et al.*, 1967). Surface counts are not usually used as these bacteria rarely grow well on an agar surface. Mara and Williams (1970) used several media to enumerate cultures of *D. vulgaris*, the most suitable being a modification of iron sulphite agar. This medium is also suitable for *D. desulphuricans* and, with extra salt, *D. salescigens*. These findings have particular relevance to the enumeration of these organisms in mixed populations where the species of the organism is unknown. There appears, as yet, to be no medium suitable for the enumeration of *Desulphotomaculum* species.

Simple three- and four-carbon compounds are generally used by sulphate-reducing bacteria as hydrogen donors; growth is, however, stimulated by complex organic material such as yeast extract, (Miller, 1949; Butlin *et al.*, 1949; Postgate, 1951a). Some strains of *D. desulphuricans* utilize oxamate (Postgate, 1953a) and choline (Hayward and Stradtman, 1959; Senez and Pascal, 1961; Baker *et al.*, 1962). Numerous other carbon compounds including carbohydrates, petroleum hydrocarbons, long chain fatty acids and alcohols have been claimed to be utilized by *Desulphovibrio*. The validity of many of these claims has been questioned by Postgate (1959a, 1965a) although it is

clear that the utilization of organic compounds may be influenced by factors such as the Eh of the medium (Grossman and Postgate, 1953). Molecular hydrogen is also a hydrogen donor for sulphate reduction by some species which possess an hydrogenase enzyme (Sisler and Zo Bell, 1951; Senez, 1955; King and Winfield, 1955; Littlewood and Postgate, 1956; Coleman, 1960; Akagi and Campbell, 1961). In general, sulphate or one of a number of other inorganic sulphur compounds is an essential requirement for the growth of sulphate-reducing bacteria but some strains are able to grow on pyruvate (Postgate, 1952a, 1963b) or fumarate (Miller and Wakerley, 1966) in "sulphate-free" media. Carbon dioxide is incorporated during the growth of *D. desulphuricans* (Sorokin, 1954a, b, 1960; Mechalas and Rittenberg, 1960; Postgate, 1960a) by mechanisms which appear similar to those present in heterotrophic organisms. Earlier reports that *D. desulphuricans* is capable of autotrophic growth on H_2, CO_2 and sulphate (Wight and Starkey, 1945; Butlin and Adams, 1947; Senez and Volcani, 1951; Adams *et al.*, 1951; Senez, 1954; Sorokin, 1954a) appear to be in error, and growth in these cases was probably due to organic impurities in the reagents used (Postgate, 1965a).

In a survey of the effects of inhibitors on sulphate-reducing bacteria, Saleh (1964) drew attention to some marked differences in the resistance of different strains to certain inhibitors, which though probably attributable in some cases to unstandardized experimental techniques, must in others reflect genuine physiological differences between strains. Saleh (1964) showed forty-five strains of sulphate-

reducing bacteria to be markedly different in their resistance to Hibitane and to a lesser degree cetyltrimethyl-ammonium bromide. Panacide was effective against all strains.

5. Rumen Metabolism of Sulphur

Protein sulphur [methionine and cyst(e)ine] is normally the main form of dietary sulphur. Methionine is believed to be an essential amino acid which, if not supplied, limits ruminant growth (Hungate, 1966; Jacobson *et al.*, 1967; McCarthy *et al.*, 1968), and it has been shown recently that an appreciable amount of this amino acid is synthesized in the rumen (Block *et al*, 1951; Conrad *et al.*, 1967). Since a large part of the dietary requirement of animals for methionine can be met by cystine or cysteine (Rose *et al.*, 1956), it was suggested early in the study of the metabolism of the sulphur amino acids that the sulphur of methionine is used in the biosynthesis of cystine.

The understanding of microbial sulphur metabolism has been simplified by the discovery of the role of enzymatically activated sulphate in sulphate metabolism, a process common to microbial, plant and animal tissues (Peck, 1962). The transformations of inorganic sulphur compounds in nature have been formalized in the so-called sulphur cycle (Wiame, 1958) that is comparable in many respects to the better known nitrogen cycle. The micro-organisms that participate in

the sulphur cycle are physiologically diverse and comprise both heterotrophic and autotrophic organisms, the latter exhibiting unique modes of existence.

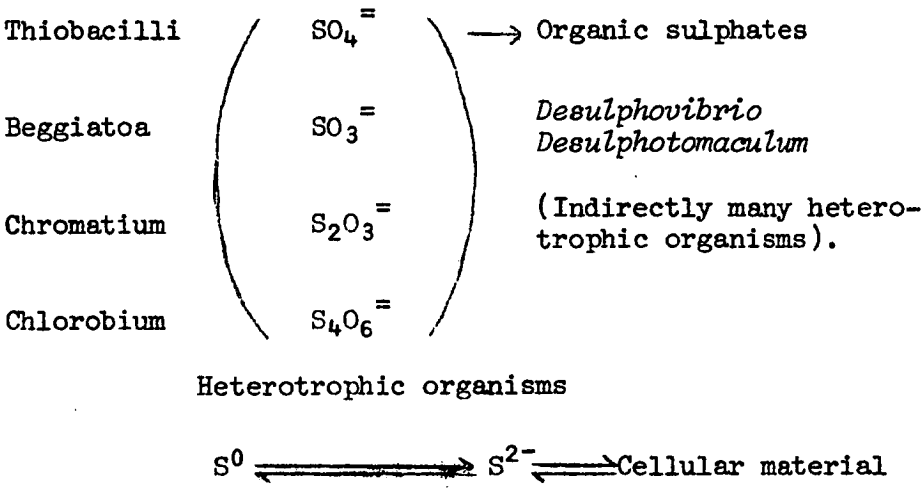


Fig. 1 Simplified diagram of the sulphur cycle. The major micro-organisms that participate in the reduction of sulphur are listed on the right hand side; those that take part in the oxidation of elemental sulphur and sulphide to sulphate are listed on the left. (from Peck, 1962)

Recent studies on the mechanism of sulphate reduction in micro-organisms indicate the existence of at least two different metabolic pathways for the reduction of sulphate (Wilson and Bandurski, 1958; Peck, 1959; Hilz *et al.*, 1959). Although many heterotrophic micro-organisms reduce sulphate, the sulphur does not usually appear as sulphide but rather is incorporated into cellular material and returned

to the sulphur cycle by other reactions. The main group of organisms producing large amounts of sulphide directly from sulphate are the sulphate-reducing bacteria.

The production of hydrogen sulphide from some inorganic and organic compounds has been frequently reported (Prevot, 1948; Clarke, 1953; Olitzki, 1954; Artman, 1956; Fuchs and Bonde, 1957; Buck and Cleverdon, 1960; Woolfolk [redacted] 1961) even though the production of sulphide directly from sulphate is not commonly observed in micro-organisms even when they are apparently reducing and incorporating sulphur into cellular materials. In the absence of sulphide production, other criteria can be employed to demonstrate that a given micro-organism can reduce sulphate to the level of sulphide and is an assimilatory sulphate reducer. In assimilatory reduction the production of sulphide from sulphate is not commonly observed in micro-organisms even when they are reducing and incorporating sulphate-sulphur into cellular material. The failure to detect sulphide under such conditions has been cited as evidence tending to rule out sulphide as an intermediate in the reduction of sulphate by these organisms.

The first step in the metabolism of sulphate is the formation of adenosine-5'-phosphosulphate (APS) and inorganic pyrophosphate (PPi) by the reaction of ATP and sulphate in the presence of the enzyme ATP-sulphurylase. The enzyme requires Mg^{++} for activity and has been characterized and purified from bakers yeast by Robbins and Lipmann (1958)a,b. The equilibrium of this reaction lies far toward ATP and sulphate ($K = 10^{-8}$, pH 8.0, 37°C) and in order to obtain large

quantities of APS the pyrophosphate must be removed with pyrophosphatase.

The two different metabolic pathways for the reduction of sulphate (Wilson and Bandurski, 1958; Peck, 1959; Hilz *et al.*, 1959) are characterized by their different enzymatic reactions:

$$ATP + SO_4^{=} \xrightleftharpoons{\text{ATP sulphurylase}} APS + PPi \qquad (1)$$

$$APS + ATP \xrightleftharpoons{\text{APS - kinase}} PAPS + ADP \qquad (2)$$

$$NADPH + PAPS \xrightleftharpoons{\text{PAPS - reductase}} NADP + PAP + HSO_3^{-} \qquad (3)$$

$$SO_3^{=} \xrightleftharpoons{\text{SO3 reductase}} S^{=} \text{ (this reaction is much simplified)} \qquad (4)$$

$$S^{=} + \text{serine} \xrightleftharpoons{\text{cysteine synthase}} \text{cysteine} \qquad (5)$$

Fig. 2 Pathway of Assimilatory Sulphate Reduction (from Peck, 1962).

The reduction first involves the formation of the sulphur-containing nucleotide, 3'-phosphoadenosine-5'-phosphosulphate (PAPS) by the combined action of two enzymes. ATP-sulphurylase catalyzes the formation of adenosine-5'-phosphosulphate (APS) and inorganic pyrophosphate from ATP and sulphate (equation 1). PAPS is then formed by the phosphorylation of APS in the 3'- position in the presence of the enzyme APS-kinase (equation 2). Finally, PAPS is reduced to sulphite and 3',5' diphosphoadenosine (PAP) as shown in equation 3.

$$H_2 + \text{cyt.}C_3\ (2Fe^{3+}) \underset{\leftarrow}{\xrightarrow{\text{hydrogenase}}} \text{reduced cyt.}C_3\ (3Fe^{++}) + 2H^+$$

$$ATP + SO_4^{=} \underset{\leftarrow}{\xrightarrow{\text{ATP-sulphurylase}}} APS + PPi$$

$$APS + \text{reduced cyt.}C_3\ (2Fe^{2+}) + 2H^+ \underset{\leftarrow}{\xrightarrow{\text{APS-reductase}}} AMP + SO_3^{=} + \text{oxid. cyt.}C_3\ (2Fe^{3+}) + H_2O$$

$$3H_2 + SO_3^{=} \underset{\leftarrow}{\xrightarrow{SO_3^{=}\ \text{reductase}}} S^{=} + 3H_2O$$

(this reaction requires cyt.C_3, hydrogenase and possibly other components. The reactions leading to cysteine formation are not clear.)

Fig. 3 Pathway of Dissimilatory Sulphate Reduction.

Hilz *et al.* (1959) showed that molybdate inhibited the activity of the first enzyme, ATP-sulphurylase, involved in the activation of sulphate. These workers demonstrated that the effect of molybdate was on the formation, and not on the reduction of PAPS by forming the substrate from labelled p-nitrophenylsulphate and PAP by a purified sulphokinase. The formation of PAPS was not inhibited by molybdate indicating that the effect of molybdate was solely on ATP-sulphurylase and that PAPS was formed as shown in Figure 2.

Three slightly different types of reaction can be catalyzed by ATP-sulphurylase (Roy and Trudinger, 1970):

1. With $SO_4^{=}$ ions, pyrophosphate exchange occurs and APS accumulates in amounts predicted from the exchange reaction. This can be represented as

$$ATP + SO_4^{=} \longrightarrow APS + PPi$$

2. With $S_eO_4^{=}$ ions, pyrophosphate exchange occurs but only traces of the anhydride can be detected, in amount far less than that expected from the pyrophosphate exchange. The reaction proceeds slowly to completion and can be represented as:

$$ATP + S_eO_4^{=} \longrightarrow APSe + PPi$$

$$AMP \qquad H_2O$$

3. With $SO_3^{=}$, $CrO_4^{=}$, $WO_4^{=}$ or $MoO_4^{=}$ ions no pyrophosphate exchange occurs and no anhydride can be detected. The reaction proceeds rapidly to completion and can be written as:

$$ATP + MoO_4^{=} \longrightarrow (APMo) + PPi$$

$$AMP \qquad H_2O$$

In this last reaction the "anhydride", if it exists at all, must have a half-life too short to allow pyrophosphate exchange to occur. However, the reaction has provided a very useful means of determining the activity of ATP-sulphurylase not only in yeast, but also in animal tissues.

Enzymatic systems are present in cell-free extracts of *D. desulphuricans* that reduce thiosulphate, sulphite and tetrathionate to sulphide with molecular hydrogen (Ishimoto *et al.*, 1958). However, early attempts to reduce sulphate with these extracts were unsuccessful. Selenate is a competitive inhibitor of the reduction of sulphate with hydrogen by whole cells; the selenate does not affect reduction of sulphite or thiosulphate (Postgate, 1949). Molybdate behaves similarly

in that it inhibits sulphate but not sulphite reduction (Ishimoto *et al.*, 1954). The suggestion that ATP-sulphurylase was involved in the reduction of sulphate by *D. desulphuricans* was put forward by Wilson and Bandurski (1958) following observations that ATP-sulphurylase catalyzes a rapid liberation of inorganic pyrophosphate from ATP in the presence of inorganic pyrophosphatase and group VI anions (selenate, chromate, molybdate, tungstate and sulphite). These workers also found that molybdate inhibited sulphate reduction but not sulphite reduction which further substantiated their belief. Cell-free extracts of *D. desulphuricans* have been shown to possess this ATP-sulphurylase since inorganic pyrophosphate is released from ATP in the presence of molybdate, tungstate and chromate (Peck, 1959). When extracts were supplemented with ATP and sulphate and placed under an atmosphere of hydrogen, hydrogen was utilized as shown in Table 2.

Table 2

Effect of ATP and molybdate on sulphate reduction with hydrogen in whole cells and cell-free extracts. (Taken from Peck, 1962)

Reaction Mixture	Activity, µl of H_2/15 min.	
	Cell-free extract	Whole cells
Complete	86	175
Minus Na_2SO_4	0	0
Minus ATP	0	314
Plus 10 µmoles molybdate	0	0

If either ATP or sulphate was omitted there was no utilization of hydrogen. ATP was not required by whole cells for the reduction of sulphate and actually seemed to inhibit the reduction. The fact that molybdate inhibited sulphate reduction in whole cells as well as in extracts, indicates that the reduction of sulphate observed in extracts is probably identical with that observed in whole cells.

Desulphotomaculum nigrificans (formerly *Clostridium nigrificans*) is a dissimilatory sulphate reducer (Campbell *et al.*, 1957) and extracts of this organism, when supplemented with ATP and $SO_4^=$ under an atmosphere of molecular hydrogen, reduce sulphate to acid-volatile sulphur. Since this reduction can be completely inhibited by $MoO_4^=$ (Peck, unpublished data cited by Peck, 1962), the first step in the reduction of sulphate by this organism seemed to be similar to that by *D. desulphuricans*, that is the formation of APS by ATP-sulphurylase (Wilson and Bandurski, 1958). In all probability, the inhibition of sulphate reduction in whole cells by group VI anions indicates that only the enzyme, ATP-sulphurylase is involved in the formation of APS. This implies that two high-energy phosphates are required for each sulphate reduced to sulphide, or each four hydrogen molecules oxidized, if it is assumed that pyrophosphate cannot give rise directly to high-energy phosphate. This implication is probably correct, because the formation of APS depends on the removal of pyrophosphate.

It has been shown that 2,4-dinitrophenol (DNP) has no effect on the reduction of thiosulphate or sulphate with hydrogen in cell-free extracts (Peck, 1960). Therefore, it can be concluded that DNP

does not have an inhibitory effect on the enzymes or electron transport sequence involved in these reductions. Since in whole cells, DNP completely inhibits the reduction of sulphate but does not affect the reduction of thiosulphate, it was apparent that DNP prevented the cells from producing the high-energy phosphate required for the reduction of sulphate but not thiosulphate. Thus, it was postulated that an oxidative phosphorylation was occurring during the oxidation of hydrogen with sulphate (Peck, 1960) and uncoupling of the oxidative phosphorylation with DNP manifested itself by failure to reduce sulphate but not thiosulphate.

Whole cells of *D. desulphuricans* can reduce DNP to amino-phenols that are much less inhibitory for sulphate reduction. When the DNP is reduced, the restoration of sulphate reduction does not occur. However, if, after the reduction of DNP, a catalytic amount of pyruvate, thiosulphate or sulphite is added, sulphate reduction is almost immediately restored to the rate observed in the controls. Since pyruvate can supply ATP by the formation of acetyl phosphate, and since sulphite and thiosulphate, by virtue of their ability to act as electron acceptors, participate in the postulated oxidative phosphorylation, it seems likely that these three compounds can supply enough ATP to initiate sulphate reduction and thereby cause this "sparking" phenomenon (Peck, 1960).

Littlewood and Postgate (1957) stated that *D. desulphuricans* is not fully permeable to anions such as sulphate and chloride. They suggested that sulphate reduction occurs either outside the

osmotic barrier or that the penetration of sulphate into the organism is regulated in such a manner that at equilibrium the ratio of the internal concentration is so low that the analytical procedure used in their work could not detect penetration of the ion even at high external sulphate concentrations. Skarzynski and Ostrowski (1958) claimed that *Thiobacillus thioparus* takes up only the outer sulphur atom of thiosulphate, leaving the inner sulphur atom of thiosulphate outside the cell as sulphate ion. These two papers give an impression that the inorganic sulphur compounds are metabolized on the surface of the cells, even though sulphate and thiosulphate are the main energy-yielding substrate for these organisms.

The suggestions of Littlewood and Postgate (1957) seem to be only partially true. Furusaka (1961) found no evidence for the idea that sulphate reduction might occur outside the osmotic barrier of the cell. The results of Furusaka showed that *Desulphovibrio* is not freely permeable to sulphate, and he concluded from his experimental findings that (1) a significant accumulation of sulphate occurs in the cell; (2) sulphate must be metabolized inside the cell; and (3) the transport of sulphate is associated with the reduction of sulphate.

It is surprising that little is known about the decomposition of organic sulphur compounds - the micro-organisms involved, the course of their dissimilation and the products formed. Methionine is one of the most significant organic sulphur compounds and it is attacked by certain bacteria and filamentous fungi with release of the nitrogen and sulphur (Akobe, 1936; Bernheim *et al.*, 1935;

Stumpf and Green, 1944; Nisman and Vinet, 1949; Fuchs and Bonde, 1957; Tsugo and Matsuoka, 1962; Starkey, 1964). The sulphur product most frequently reported is methanethiol. Other products are dimethyl disulphide (Challenger and Charlton, 1947; Kallio and Larsen, 1955; Tsugo and Matsuoka, 1962), dimethyl sulphide (Challenger and Charlton, 1947), ethyl sulphide (Akobe, 1936), hydrogen sulphide (Challenger and Charlton, 1947; Stahl *et al.*, 1949; Tsugo and Matsuoka, 1962) and sulphate (Garrean, 1941; Stahl *et al.*, 1949). The two sulphur products arising from microbial decomposition of methionine (Segal and Starkey, 1969), methanethiol and dimethyl disulphide, are volatile and escape from the media resulting in a decrease in the sulphur content proportional to the amount of methionine decomposed.

EXPERIMENTAL SECTION A

THE ISOLATION OF ADENOSINE-5'-PHOSPHOSULPHATE AND 3'-PHOSPHOADENOSINE-5'-PHOSPHOSULPHATE FROM THE RUMEN OF SHEEP.

Introduction

Positive responses to sulphur supplementation of ruminant rations is dependent on the total sulphur and nitrogen concentration of the diet and their proportions and to a lesser extent the form of dietary sulphur. The ascribed reason for such sulphur responses is the satisfaction of rumen microbial sulphur requirements for growth and metabolism with consequent increases in the production of microbial protein (Hume and Bird, 1970) allied with an increased fermentation of dietary components resulting in increased digestibility of rations (Williams and Moir, 1951; Bray and Hemsley, 1969).

Since the sulphur component of sulphur amino acids is in the reduced sulphide state, forms of dietary sulphur of higher oxidation states require prior reduction before synthesis takes place. Peck (1962) has proposed that the bacterial reduction of sulphate to sulphide may proceed by one of two biochemical pathways.

"Sulphate reduction that is physiologically characterized by the formation of only enough sulphide to meet nutritional requirements has been termed assimilatory reduction. This is the only type of sulphate reduction occurring in plants and the most commonly encountered type in

bacteria. A small group of anaerobic bacteria, known as the sulphate-reducing bacteria, produce massive amounts of sulphide during growth in the presence of sulphate and an electron donor. This process has been identified as dissimilatory or respiratory sulphate reduction in that sulphate serves as the terminal electron acceptor in respiration in much the same manner as oxygen in aerobic respiration." (Taken from Peck, 1970)

The distinguishing features between these two pathways is that the dissimilatory group use the activated sulphate APS as the intermediate substrate for reduction whereas further phosphorylation of APS to form PAPS is required before sulphate reduction takes place in the assimilatory group.

Indirect evidence suggests that both sulphate reduction pathways exist in the rumen microbial system since Lewis (1954), Anderson (1956) and Bray (1964) have shown that sulphate is reduced to free sulphide in the rumen suggesting the dissimilatory pathway is present. Other workers (Emery *et al.*, 1954) have demonstrated the ability of certain rumen bacterial species to grow in media where sulphate was the sole sulphur source. In the situation where sulphate is the sole source of dietary sulphur other bacteria which lack the ability to reduce sulphate (Coleman, 1960) are dependent on either the dissimilatory bacteria or the breakdown of sulphur amino acids for their sulphide requirements. Thus while the existence of these two sulphate reduction pathways in the rumen system is suspected, direct evidence of their existence is lacking and these studies were commenced with the object of looking for

the presence and studying the metabolism of APS and PAPS in the rumen of sheep fed inorganic sulphate as their major dietary sulphur source.

Materials and Methods

Three mature crossbred wethers fitted with permanent rumen fistulae were kept in metabolism crates and offered 800 gm dry matter daily at 9 a.m. Over a period of a month the sheep were conditioned to consume the full ration within an hour of feeding.

The basal diet contained less than 0.1 per cent total sulphur to which was added 1.5 per cent sulphate as anhydrous sodium sulphate. The composition of the diet is shown in Table 3.

Table 3

Composition of experimental diet (% dry matter)

Component	
Oaten chaff	95.8
Urea	2.4
Na_2SO_4	1.5
Trace element mix*	0.3

* Trace element mix of Moir and Harris (1952).

Experimental Programme

1. The Isolation of APS and PAPS from the Rumen

The experiment was run for a period of four weeks during which time each sheep was sampled weekly. On the day of sampling each sheep, after full feed consumption had occurred, was given 4 gm of sodium sulphate to which was added 0.5 mCi of S^{35}. Rumen samples were taken every five minutes for 25 minutes following introduction. The samples were filtered through surgical gauze and then buffered with N potassium hydrogen phosphate at the rate of 15 ml of $KHPO_4$ per 45 ml of strained rumen liquor. Following this operation 5 ml of cold ethanol was added to stop further metabolism (Still, *pers. comm.*), 15 ml aliquots of each solution were subjected to Sonic oscillation for 10 minutes to disrupt bacterial cells. The particulate matter was then removed by centrifugation at 9000 R.P.M.

Adenosine-5'-phosphosulphate (APS) was extracted from the supernatant by the method of Baddiley *et al.* (1957) and 3'-phospho-adenosine-5'-phosphosulphate (PAPS) was extracted by the method of Robbins and Lipmann (1958). Both were isolated and identified chromatographically using chemically prepared APS (Baddiley *et al.*, 1957) and PAPS (Robbins and Lipmann, 1958) as standards.

Inorganic sulphate was determined by the method described by Bray (1969) using the colour reaction of Dean (1966), on rumen samples taken every 5 minutes. Two per cent sodium bicarbonate was used to minimise diffusion losses as mentioned by Bird and Fountain (1970).

A nuclear Chicago model liquid scintillation counter was used to measure radioactivity. The distribution of radioactivity on the chromatograms was determined by cutting vertical strips of chromatography paper into horizontal sections of one centimetre. These were then counted in the diotol scintillant of Herberg (1960).

2. *In vitro* Measurement of Sulphide Production from APS^{35} and $PAPS^{35}$.

Chemically prepared solutions of S^{35}-labelled APS and PAPS (Baddiley *et al.*, 1957; Robbins and Lipmann, 1958) were added to an *in vitro* rumen fermentation system similar to that described by Barnett and Reid (1961). The rumen liquor used was taken from sheep fed on a diet containing sodium sulphate as the sole source of sulphur. 250 ml of strained rumen fluid were added to the *in vitro* system along with 2 ml of either chemically prepared APS^{35} or $PAPS^{35}$. The sulphide produced was blown over by the nitrogen and carbon dioxide gas (95% N_2: 5% CO_2) and caught in the absorbing solution of 1 N NaOH and measured by the method of Bray (1969) using the colour reaction of Dean (1966).

Rumen sulphide radioactivity was measured by placing 0.5 ml of the absorbing solution used to catch rumen sulphide for chemical estimation into 15 ml of diotol and counting by liquid scintillation. Quenching was corrected by the use of internal standards. The counting efficiency of the Nuclear Chicago Model Scintillation counter was 75 per cent.

Results

The concentrations of rumen sulphate over 25 minutes following the introduction of S^{35}-labelled sodium sulphate are presented in Table 4.

Table 4

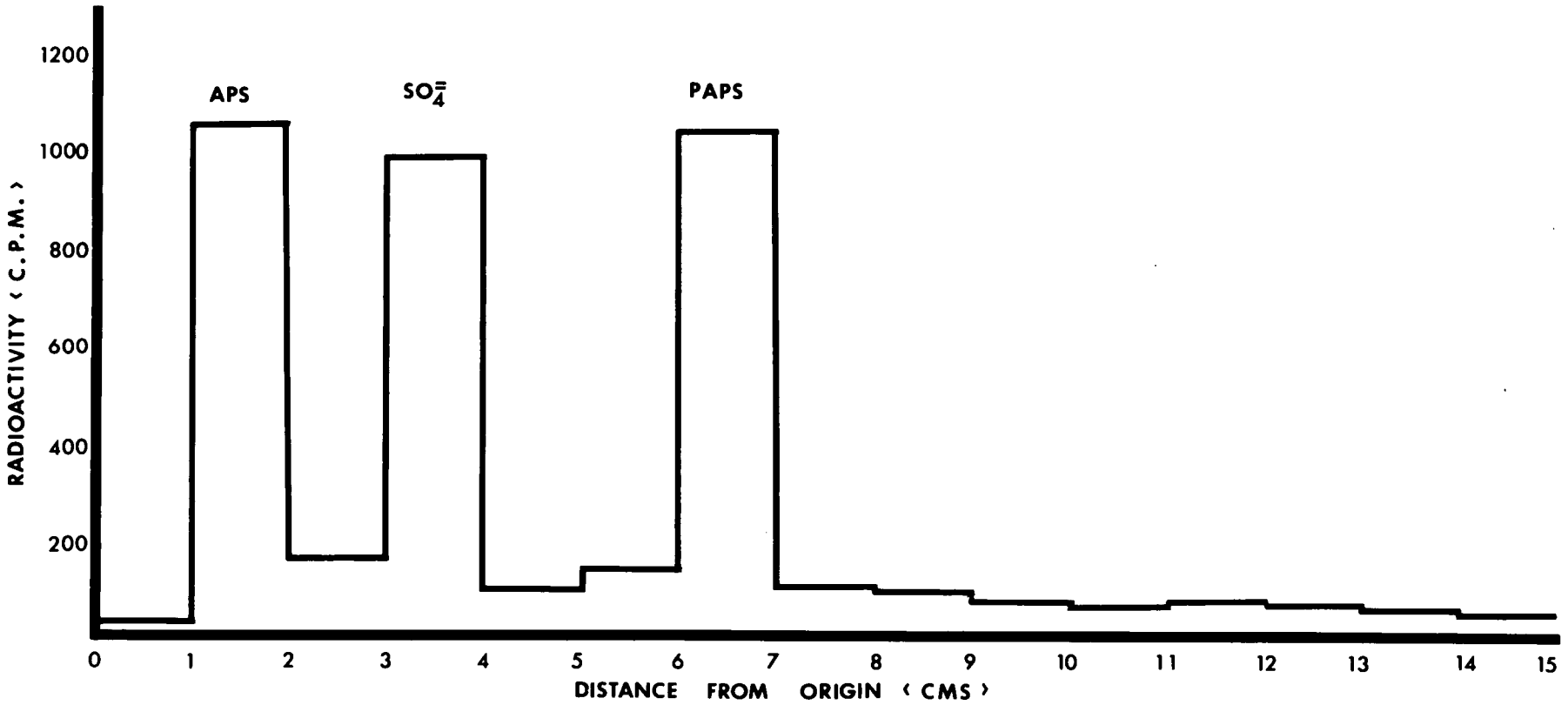
The mean concentration of inorganic sulphate in rumen fluid measured at 5 minute intervals after the administration of 4 gm of sodium sulphate given one hour after feeding.

Sheep Number	Time (minutes) T5	T10	T15	T20	T25
	Inorganic sulphate (µg S/ml)				
1	297	200	124	94	90
2	106	74	56	54	54
3	326	83	49	49	49

As can be seen in Table 4 the concentration of sulphate in the rumen declined with time in all sheep ($P < 0.05$). Even though the same amounts of sulphate (4 gm) were added to the rumen of each sheep there was a significant difference between sheep in initial rumen sulphate levels ($P < 0.01$).

An examination for the presence of APS and PAPS in rumen samples taken at 5 minute intervals from T0 to T25 was carried out. A typical example of the distribution of radioactivity along the chromatogram can be seen in Figure 4. This figure represents the

Fig. 4 Chromatogram showing the isolation of both APS and PAPS from the intact rumen of sheep 1 at time T10. The distance moved by the solvent front is shown in centimetres and the Rf values - 0.11 for APS, 0.20 for SO_4 and 0.38 for PAPS are also shown.


1200
1000
800
600
400
200
APS
$SO_4^{=}$
PAPS
RADIOACTIVITY (C.P.M.)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
DISTANCE FROM ORIGIN (CMS)

chromatogram of sheep 1 at T10, but is typical of all three sheep. The mean values of these data are presented in Table 5.

Table 5

The mean radioactivity components separated chromatographically from rumen fluid taken from sheep at 5 minute intervals following the administration of 4 gm of S^{35}-labelled sodium sulphate.

		Radioactivity levels determined at five minute intervals (cpm).				
Time (minutes)		T5	T10	T15	T20	T25
Rf values:	0.11	439	770	542	373	207
	0.20	4531	650	416	245	102
	0.38	676	851	614	394	136

The Rf values for chemically prepared S^{35}-labelled APS and PAPS were 0.11 and 0.38 respectively and S^{35}-sulphate was 0.20. These values were of the same magnitude as those reported in the literature (Baddiley *et al.*, 1957; Robbins and Lipmann, 1958). From a comparison of the distribution of radioactivity on the "rumen liquor" chromatograms and those of the standards it would appear that both S^{35}-labelled APS and PAPS were present in the rumen liquor. Further the maximum radioactivity exhibited by APS and PAPS in the rumen occurred in samples taken 10 minutes after the introduction of S^{35}-labelled sodium sulphate into the rumen. As can be seen in Table 4 the most rapid decline in sulphate radioactivity occurred between T5 and T10

which would substantiate the hypothesis that sulphate is reduced via APS and PAPS in the rumen.

The S^{35}-labelled APS and PAPS which had been chemically prepared were incubated independently with rumen liquor in an *in vitro* system, and sulphide production from this system was measured. These results are presented in Table 6.

Table 6

The production of sulphide (μg S^{2-} sulphur/ml incubation media) and sulphide radioactivity (counts per minute) collected following the incubation of S^{35}-labelled APS and PAPS with rumen liquor.

	Time of sampling (mins)									
	30	60	90	120	150	180	210	240	270	300
APS^{35}										
μg S^{2-}/ml rumen fluid	1.2	1.6	2.4	3.4	3.1	3.1	3.2	3.8	4.0	1.7
Radioactivity (cpm)	384	453	612	811	723	676	604	645	843	347
Relative activity (cpm/μg S^{2-})	320	283	255	239	233	218	189	170	211	204
$PAPS^{35}$										
μg S^{2-}/ml rumen fluid	1.3	2.7	4.2	4.8	4.1	3.6	3.8	1.6	0.6	0.2
Radioactivity (cpm)	298	635	950	1010	924	617	641	450	103	52
Relative activity (cpm/μg S^{2-})	229	235	226	210	225	171	169	281	171	260

There was an increase in sulphide production from T0 to T270 when APS^{35} was incubated in the *in vitro* system. The relative activity of the system did not change significantly throughout the 300 minutes of incubation. The $PAPS^{35}$ produced increasing amounts of sulphide and radioactivity from T0 to T150, and then the level of sulphide declined to T300. The relative activity did not change specifically. Under normal circumstances $PAPS^{35}$ would not be expected to produce large quantities of sulphide.

Discussion

The isolation of both APS and PAPS from the rumen indicated that the pathway of sulphate reduction as described by Peck (1959, 1962) was most likely present. The two-step biosynthesis of active sulphate was suggested by this isolation. However, such data does not show if the APS and PAPS systems are the main means of sulphate reduction in the rumen. It does indicate, however, that at least the assimilatory pathway of sulphate reduction is present, because assimilatory sulphate reduction is a property of the majority of bacteria, fungi, yeasts, algae and plants. In the experiments reported here no attempts were made to isolate the specific bacteria responsible for assimilatory or dissimilatory reduction of sulphate.

Sulphate reduction in the rumen is a rapid process, the bulk of the sulphate disappearing within 30 minutes following the introduction of S^{35}-labelled sodium sulphate into the rumen. Table 4 shows

that the concentration of sulphate in the rumen declined from a level of 243 µg sulphate-sulphur per ml of rumen fluid five minutes after the addition of 4 g sodium sulphate to a level of 64 µg 25 minutes later, presumably due to reduction to sulphide since sulphate is readily reduced to sulphide in the rumen as demonstrated by Lewis (1954), Anderson (1956), Bray (1969a) and *in vitro* by Henderickx (1961). Bray (1969b) studied the disappearance of sulphide from the rumen and stated that three avenues of loss seemed possible, viz. passage across the rumen wall, passage down the alimentary tract, and oxidation of the sulphide ion. Bray's data support the hypothesis that absorption from the rumen is the main factor responsible for the fall in rumen sulphide levels. In contrast to the rapid fall of sulphide concentration Bray (1969b) found the levels of S^{35}-labelled sulphate in the rumen were remarkably stable.

In the present experiments the reduction of sulphate to sulphide must be responsible for the rapid decline shown in Table 4 since Bird and Hume (1971) found that the flow of sulphate and sulphide to the omasum was less than 10 per cent of the total sulphur flow regardless of the form or the level of supplemental sulphur.

The between sheep differences in rumen sulphate levels as shown in Table 4 were not caused by dietary intake since intakes were all the same. However, differences in rumen volumes and differences in the rate of digestion were not calculated and these between sheep differences may have accounted for some of the variation.

The changing pattern of rumen sulphate concentration was used to predict the time when APS and PAPS would most likely be present on the premise that the disappearance of sulphate was due to the reduction of sulphate to sulphide. The mean values of radioactivity within the rumen fluid supernatant (Table 5) show that APS^{35} reached a maximum concentration at T10, and $PAPS^{35}$ was a maximum at T10. The PAPS reached a greater concentration within the rumen but no assumptions as to the likelihood of the assimilatory or dissimilatory being the dominant one could be made from such a result. Both S^{35}-labelled APS and PAPS exhibited the maximum radioactivity at T10 which is coincident with the period of maximum sulphate disappearance (Table 4). These two lots of data support the hypothesis that sulphate was reduced via APS and PAPS in the rumen.

In vitro, sulphide was produced in large quantities by the micro-organisms incubated with both APS^{35} and $PAPS^{35}$. The peak of sulphide production from PAPS occurred at T120 and then declined to almost zero by T300, while sulphide production from APS didn't peak until T270. The $PAPS^{35}$ produced higher levels of sulphide initially but at T300 almost all the $PAPS^{35}$ had disappeared. This was similar to the situation within the rumen in which the PAPS disappeared more rapidly than the APS. The production of free sulphide would not normally be expected in the case of PAPS since it is thought to be only a substrate for assimilatory sulphate reduction (Peck, 1962). Robbins and Lipmann (1958) presented evidence about the kinetics of the reaction

$$APS + ATP \underset{}{\overset{\text{APS-kinase}}{\rightleftharpoons}} PAPS + ADP$$

from which they suggested the reaction was analogous to the hexokinase reaction where they estimated the reaction had a $\Delta F'$ of -6000 calories/mole (pH 8, 37°C), which indicates that the reaction equilibrium lies strongly in the direction of PAPS. However, the removal of either APS or ATP would drive the reaction to the left leading to the formation of APS from PAPS. In our results high levels of sulphide were produced from both APS^{35} and $PAPS^{35}$ indicating that PAPS was probably converted to APS during the reduction process. The relative activity figures (Table 6) for both APS^{35} and $PAPS^{35}$ did not differ significantly lending support to the above hypothesis. However, the direct reduction of PAPS to produce sulphide can not be discounted.

The small variation in relative activity of sulphide from both the incubations indicates that the sulphide production was from the labelled sulphate pool. However evidence is not sufficient to evaluate the contribution of assimilatory or dissimilatory sulphate reduction although it could be argued that free sulphide production from PAPS was dissimilatory in nature and thus the major pathway was dissimilatory.

EXPERIMENTAL SECTION B

The Effects of Group Six Anions on the Production of Sulphide in the Rumen of Sheep.

The studies reported in this section cover five feeding experiments, viz:-

1. The initial investigations involving group VI anions and their effects on sulphide production.
2. The effects of four levels of dietary molybdenum - 40, 240, 720 and 1440 ppm - on the production of sulphide in the rumen.
3. A comparison of the effects of diets with and without added sulphate, and with and without added molybdenum on the production of sulphide in the rumen, and on other functions of general rumen metabolism.
4. The effects of a further six levels of dietary molybdenum - 20, 48, 76, 104, 132 and 160 ppm - on the production of sulphide in the rumen and on other functions of general rumen metabolism.
5. The effects of molybdenum on sulphate reduction *in vitro* using a pure culture of *Desulphovibrio desulphuricans* and a sulphate-reducing bacteria isolated and enriched from the sheep rumen.

Introduction

The experiments reported in section A cover the isolation of both APS and PAPS from the sheep's rumen. The original intention was to isolate the enzymes involved in the sulphate-reductive pathway, in particular APS-reductase and PAPS-reductase. However, although initial attempts were made to isolate these enzymes, they proved unsuccessful and so it was decided to investigate the effects of the group VI anions on the reduction of sulphate in the rumen. The group VI anions - sulphite, tungstate, selenate, chromate and molybdate - competitively inhibit the formation of APS from inorganic sulphate and ATP as discussed in the literature review and in section A. In view of the inhibitory characters of these anions on sulphate reduction the experiments reported in this section were initiated to gain further evidence to support the existence of enzymatic sulphate reduction in the rumen.

The final section of experimental work reported describes the tolerance to various concentrations of molybdenum judged by the sulphide production of sulphate-reducing bacteria isolated from the rumen, and the common sulphate-reducing bacteria *Desulphovibrio desulphuricans*.

ANALYTICAL PROCEDURES

(a) Collection

Rumen samples were collected per fistulam from day 11 to day 14 inclusive of each feeding period in section B(1), day 4 to day 7 inclusive in sections B(2) and B(4), and day 2 to day 7 inclusive in section B(3).

Urine and faeces were collected on days 1 to 7 inclusive of the collection period. Faeces were collected in a canvas bag attached to a light leather body harness. The faeces were weighed and dried at 100°C to determine dry matter, and 10 per cent samples were stored in screw-top jars. Urine was collected in a container bottle to which was added 10 ml concentrated HCl to prevent loss of ammonia. The volume of urine at each collection time was measured, made up to 2000 ml with distilled water and a 10 per cent aliquot bulked for later measurements.

(b) Sample Analysis

Rumen sulphate and rumen sulphide levels were determined on strained rumen fluid samples by the methods of Bray (1969a) using the colour reaction of Dean (1966). All sulphide determinations were commenced within two minutes of extracting the rumen fluid to minimise oxidation and diffusion losses.

Rumen volatile fatty acids were determined by the method of Barcroft *et al.* (1944). The *in vitro* rumen technique used for the determination of sulphide production was similar to that described by Barnett and Reid (1961). Rumen pH was measured with a Philips GA110 pH electrode and rumen redox potentials with a Philips MQ13 platinum electrode. The cotton thread digestion technique was that of Hemsley and Moir (1963).

Samples of feed, urine and faeces were analyzed for total sulphur by the method of Bray (1969a) using the colour reaction of Dean (1966), and for total nitrogen by the Kjeldahl method. Total molybdenum levels were determined by atomic absorption spectrometry (Lamp, 1969) following digestion with perchloric acid.

(c) Microbiological Methods

Total rumen bacteria were estimated on rumen samples collected 8.5 hours post-feeding by the culture method of Munch-Peterson (1964). Sulphate-reducing bacteria were estimated by the most-probable number technique using the medium in Table 6 which has been modified from that of Butlin, Adams and Thomas (1949).

Table 6

Medium used for isolation of sulphate-reducing bacteria from rumen fluid of sheep.

Component	g/litre	Component	g/litre
K_2HPO_4	0.5	Sodium lactate	6.0
NH_4Cl	1.0	Yeast extract	1.0
Na_2SO_4	3.8	Tri-sodium citrate	5.0
$CaCl_2$	0.1	Ferric citrate	1.0
$MgSO_4.7H_2O$	2.0	Distilled water	1 litre

The ferric citrate was dissolved in boiling water and then the remainder of the salts were dissolved. The reaction was adjusted to pH 7.4-7.5 with sodium hydroxide, and dispensed in 8 ml aliquots in 12 x 100 mm screw-capped culture tubes and autoclaved for 20 minutes at 15 pSi. The culture tubes were incubated in McIntosh and Fildes jars with an atmosphere of hydrogen enriched with carbon dioxide provided by a BBL Gas Pak.

Section B(1)

THE INITIAL INVESTIGATIONS INVOLVING GROUP VI ANIONS AND THEIR EFFECTS ON SULPHATE REDUCTION.

Materials and Methods

(a) Experimental and Diet

Two mature cross-bred wethers fitted with permanent rumen fistulae were kept in metabolism crates and fed one of the rations listed in Table 7, so that over the course of the experiment each sheep received each treatment.

Table 7

Composition of experimental diets (percentage dry matter).

Component	Diets					
	Basal	A	B	C	D	E
Oaten chaff	95.11	95.11	95.11	95.11	95.11	95.11
Urea	2.1	2.1	2.1	2.1	2.1	2.1
Trace element mix*	0.3	0.3	0.3	0.3	0.3	0.3
Na_2SO_4	1.5	1.5	1.5	1.5	1.5	1.5
$Na_2MoO_4.2H_2O$	-	$6x10^{-3}$	-	-	-	0.19
Na_2SeO_4	-	-	0.19	-	-	-
Na_2WO_4	-	-	-	0.19	-	-
Na_2CrO_4	-	-	-	-	0.19	-
Daily group VI anion intake (g)	-	0.05	1.54	1.54	1.54	1.54

*Trace element mix of Moir and Harris (1962) with the molybdenum component omitted.

The differences in sulphide concentration in the rumen digesta between basal and the other diets was taken as a criterion of group VI anion effects on sulphate reduction.

The experimental periods were divided into a 14 day period on the basal ration followed by a 14 day period on one of the group VI anion treatments. This experimental design was chosen because:

(1) Normal rumen sulphide levels were established for each sheep in the second 14 day phase of the basal alone treatment.

(2) A 14 day basal ration treatment before the group VI anion treatment was to ensure that rumen sulphide levels had returned to normal levels before the anion was fed. During this period each sheep was given 8 mg copper (as $CuCl_2.2H_2O$) per day to restore possibly depleted body-copper stores. Eight hundred grams dry matter were offered daily at 9 a.m. which was consumed in all cases by 10 a.m.

(b) Experimental Programme

The experimental programme for these preliminary experiments is set out in Table 8. The sodium salts of these anions were used in all cases.

Table 8

Summary of experimental treatments used in the preliminary investigations of the effect of group VI anions on sulphate reduction within the rumen.

Experiment number	No. of sheep	Anion used	Amount of anion added (g per day)
1	2	nil	nil
2	2	MoO_4^{2-}	1.54
3	2	MoO_4^{2-}	0.05
4	2	nil	nil
5	2	WO_4^{2-}	1.54
6	2	nil	nil
7	2	CrO_4^{2-}	1.54
8	2	nil	nil
9	2	SeO_4^{2-}	1.54
10	1	Cl	1.54
11	1	HCO_3^-	1.54

Results

A substantial inhibition of sulphide production occurred for all group VI anion treatments (with the exception of 50 mg of MoO_4 per day) (see Table 9).

When 50 mg of MoO_4 per day was fed a slight increase in the production of rumen sulphide as compared to the basal ration was observed from T0 to T60, but from T90 to T300 they were below those of the basal ration, but substantially greater than those of the group VI anions fed at the rate of 1.54 gm per day.

Table 9

The effect of dietary group VI anions on the concentration of sulphide sulphur in the rumen fluid of sheep (means of two sheep).

Sampling time (mins after feeding)	Rumen sulphide concentration (μg S/ml)					
	basal	MoO_4^{2-} *	MoO_4^{2-} **	WO_4^{2-} *	CrO_4^{2-} *	SeO_4^{2-} *
0	1.2	1.2	1.3	1.3	1.3	1.1
30	1.4	0.3	1.9	0.3	0.5	0.4
60	1.5	0.5	1.8	0.4	0.5	0.2
90	2.0	0.5	1.9	0.4	0.3	0.2
120	2.4	0.4	1.8	0.5	0.4	0.3
150	2.6	0.5	1.8	0.5	0.4	0.3
180	2.5	0.3	1.9	0.3	0.3	0.5
210	2.6	0.4	1.5	0.3	0.5	0.3
240	1.9	0.5	1.3	0.3	0.4	0.2
270	2.4	0.4	1.2	0.4	0.4	0.2
300	1.9	0.4	1.2	0.3	0.3	0.2

** 50 mg of trace element per day fed in the ration
* 1.54 g of trace element per day fed in the ration.

Although the effect on rumen sulphide production was evident, it was not possible at this stage to state whether the effect was due to the group VI anions or the sodium cation. A further two experiments were designed (experiments 10 and 11 from Table 8) using the salts of sodium chloride and sodium bicarbonate to test the effect of sodium on the inhibition of sulphate reduction. The sulphide levels produced when

these two salts were incorporated in the diet at similar levels of Na as those used with the group VI anions are given in Table 10.

Table 10

The effect of dietary sodium on the concentration of sulphide sulphur in the rumen fluid of sheep.

Sample time (mins after feeding)	Rumen sulphide concentration (µg S/ml)		
	basal	NaCl	$NaHCO_3$
0	1.1	1.1	1.0
30	1.3	1.1	1.3
60	1.3	1.3	1.5
90	1.3	1.1	1.3
120	1.6	1.5	1.7
150	1.9	1.4	1.5
180	1.5	1.7	1.5
210	1.4	1.7	1.5
240	1.7	1.9	1.3
270	2.1	2.3	1.7
300	2.0	2.0	1.8

There was no significant difference between the basal level and the two different sodium salts in the level of sulphide production, indicating that the inhibition of sulphide production observed with group VI anions was in fact due to the anion and not to the common sodium cation.

The concentrations of steam volatile fatty acids were measured at each sampling time (see Table 11).

Table 11

The effect of group VI anions fed in the ration on the concentrations of steam volatile fatty acids in the rumen fluid of sheep (means of two sheep).

Sample time (mins after feeding)	Rumen steam volatile fatty acid concentration (m Moles/litre)					
	basal	MoO_4^{2-} *	MoO_4^{2-} **	WO_4^{2-} *	CrO_4^{2-} *	SeO_4^{2-} *
0	57.0	55.0	56.0	56.0	55.5	55.5
60	50.5	48.5	49.5	52.5	52.0	51.5
120	60.0	58.5	58.0	57.5	56.0	57.0
180	56.5	57.5	58.5	58.0	58.5	58.0
240	54.5	52.5	53.0	53.5	53.0	53.5
300	47.0	49.0	49.5	49.5	49.0	48.5

** 50 mg molybdenum per day fed in the ration

* 1.54 g molybdenum per day fed in the ration.

There was no significant difference in VFA production between the basal diet and any of the other diets containing group VI anions which would suggest that normal rumen fermentation was not disturbed by the addition of group VI anions.

The *in vitro* rumen technique was used to determine the effects of Panacide on the sulphate-reducing bacteria (see Table 12).

When the Panacide was added an immediate effect was noticed, and the production of sulphide declined rapidly when compared to the basal level.

Table 12

The effects of adding Panacide on sulphide production (μg S/ml rumen fluid) in an *in vitro* rumen system 90 minutes after the commencement of sampling.

Sample time (mins)	Sulphide (μg S/ml)	
	basal	with Panacide
30	2.4	2.9
60	2.9	2.4
90	3.4	4.6
120	2.7	1.5
150	4.2	1.3
180	3.5	0.9
210	4.3	0.4

The level of sulphide continued to decline with time following addition of Panacide until T210, while the basal level maintained a relatively constant level of sulphide production over the same time interval. The Panacide had no effect on bacteria other than the sulphate-reducing bacteria in the *in vitro* system.

Sections B(2), B(3) and B(4)

THE EFFECT OF DIETARY MOLYBDENUM ON THE REDUCTION OF INORGANIC SULPHATE IN THE RUMEN.

In experiments B(2) and B(3) four mature cross-bred wethers and in experiment B(4) six mature cross-bred wethers all fitted with permanent rumen fistulae were kept in metabolism crates over the course of the experiments.

Experiment B(2)

The sheep in experiment B(2) were fed a basal ration of oaten chaff, urea, trace element mix and anhydrous sodium sulphate similar to that presented in Table 7. Four levels of dietary molybdenum were fed in addition to this basal diet (see Table 13). The experimental periods were divided in the same way as described in section B(1).

This experiment was designed to determine the effects of four levels of dietary molybdenum ranging from 40 ppm to 1440 ppm on the production of rumen sulphide following the initial investigations in which 50 mg per day of MoO_4 was found to increase the level of rumen sulphide in the initial stages of sampling. The sheep were fed the basal ration plus four levels of added sodium molybdate in a 4 x 4 Latin square design. Each sheep was offered 1200 gm dry matter per day. Full feed consumption occurred with all diets except for sheep 2 on the 720 ppm molybdenum diet. Corrections were made for the lower intakes

of sheep 2 in statistical treatments by a missing plot technique.

Table 13

Dietary molybdenum treatments used in experiment B(2).

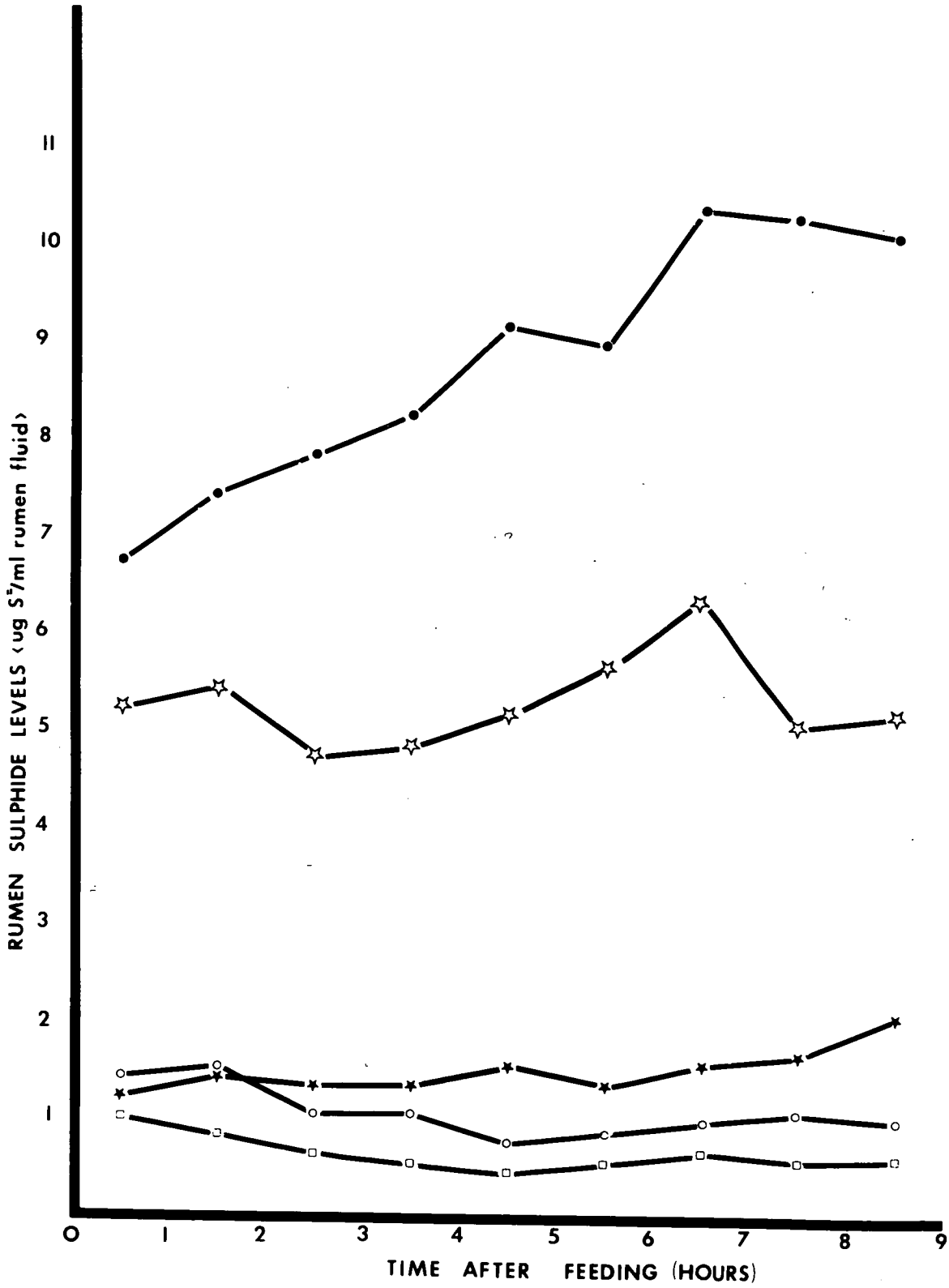
Component	Diets				
	basal	A	B	C	D
$Na_2MoO_4.2H_2O$	0	4.2×10^{-4}	2.5×10^{-3}	7.5×10^{-3}	1.5×10^{-2}
Mo-content (ppm)	< 0.7	40	240	720	1440
Daily Mo intake (mg)	< 0.9	50	300	900	1800
Daily S intake (g)	5.9	5.9	5.9	5.9	5.9

Results

The effects of varied dietary levels of molybdenum on the reduction of inorganic sulphate within the rumen were measured by the production of rumen sulphide (see Figure 5). The control level was that established when the sheep were fed the basal ration without molybdenum.

There was a varied effect of molybdenum on rumen sulphide concentration. A dietary level of 40 ppm molybdenum enhanced sulphide production above that of the control ($P < 0.05$) whereas higher dietary levels (240, 720 and 1440 ppm) severely depressed sulphide production.

Fig. 5 Sulphide concentration in the rumen of sheep fed diets containing molybdenum at the following concentrations: <0.7 ppm ✱——✱; 40 ppm •——•; 240 ppm ×——×; 720 ppm ○——○; 1440 ppm □——□ (means of four sheep).


RUMEN SULPHIDE LEVELS (ug S⁼/ml rumen fluid)
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

The concentration of molybdenum in the rumen was positively correlated to dietary molybdenum concentration and the variation of rumen molybdenum with time for each treatment is shown in Figure 6. An unusual aspect of these results was that the rumen molybdenum concentrations remained constant over the 8.5 hour sampling period.

There was no significant effect of varied dietary levels of molybdenum on pH, volatile fatty acid concentration or rumen redox potentials as shown in Figures 7, 8 and 9.

The digestion times for cotton thread bundles, and the total numbers of rumen bacteria determined during each treatment are presented in Table 14.

Table 14

The effect of dietary molybdenum on the digestion time of cotton thread bundles in the rumen and on the total numbers of rumen bacteria measured 8.5 hours after feeding (means of four sheep).

Treatment	Hours taken for 50% weight loss of cotton thread	Total bacteria per ml rumen fluid
basal	40.5	3.5×10^{10}
A	37.5	1.6×10^{10}
B	37.0	7.9×10^{9}
C	38.0	4.8×10^{10}
D	40.0	3.8×10^{10}

Fig. 6 Molybdenum concentration in the rumen of sheep fed diets containing molybdenum at the following concentrations: 40 ppm ●—● ; 240 ppm ×—× ; 720 ppm ○—○ ; 1440 ppm ◘—◘ (means of four sheep).

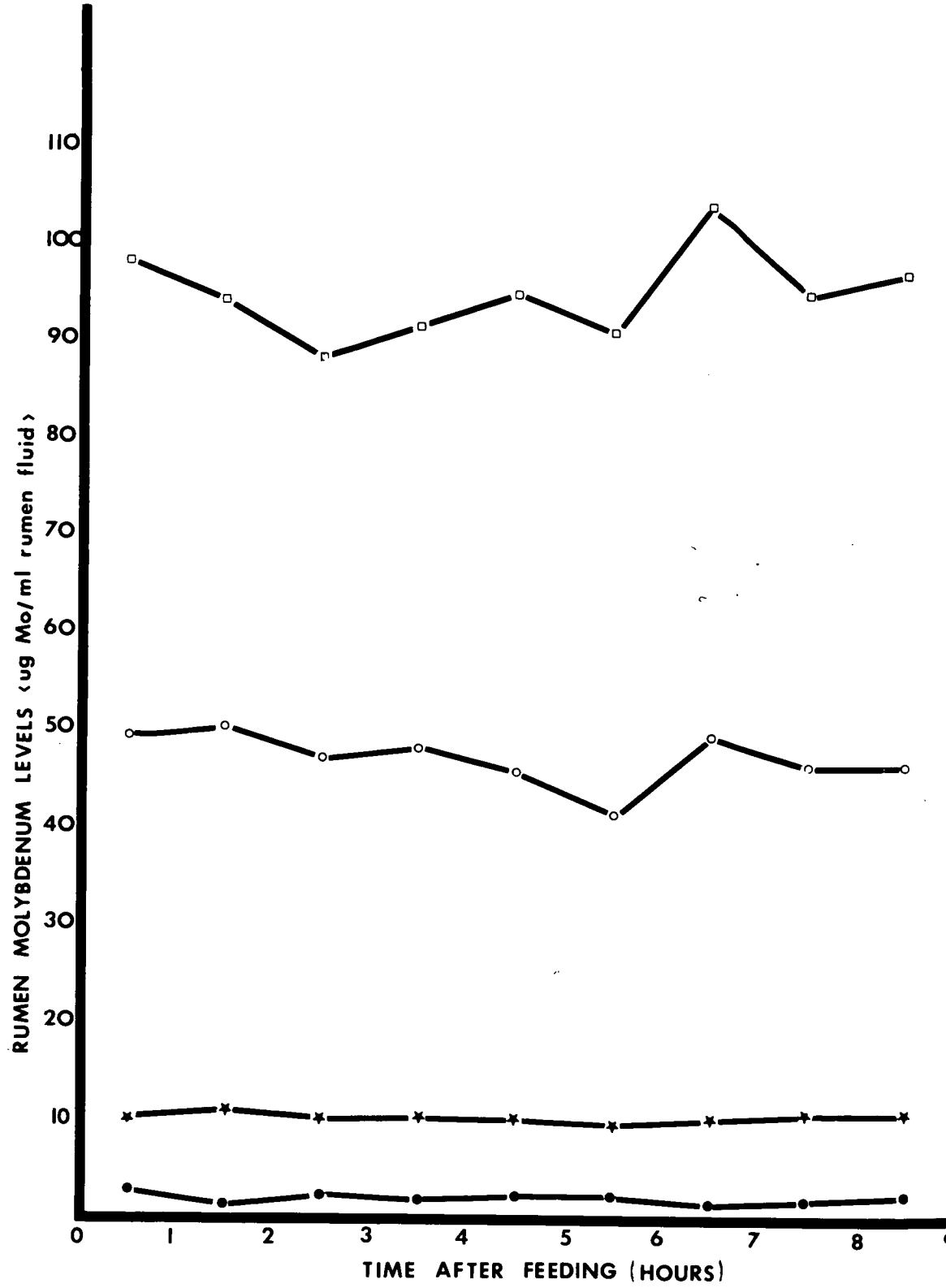

RUMEN MOLYBDENUM LEVELS (ug Mo/ml rumen fluid)
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

Fig. 7 The effect of dietary molybdenum on rumen pH in sheep fed four levels of molybdenum, namely 40 ppm, 240 ppm, 720 ppm and 1440 ppm (means of four sheep).

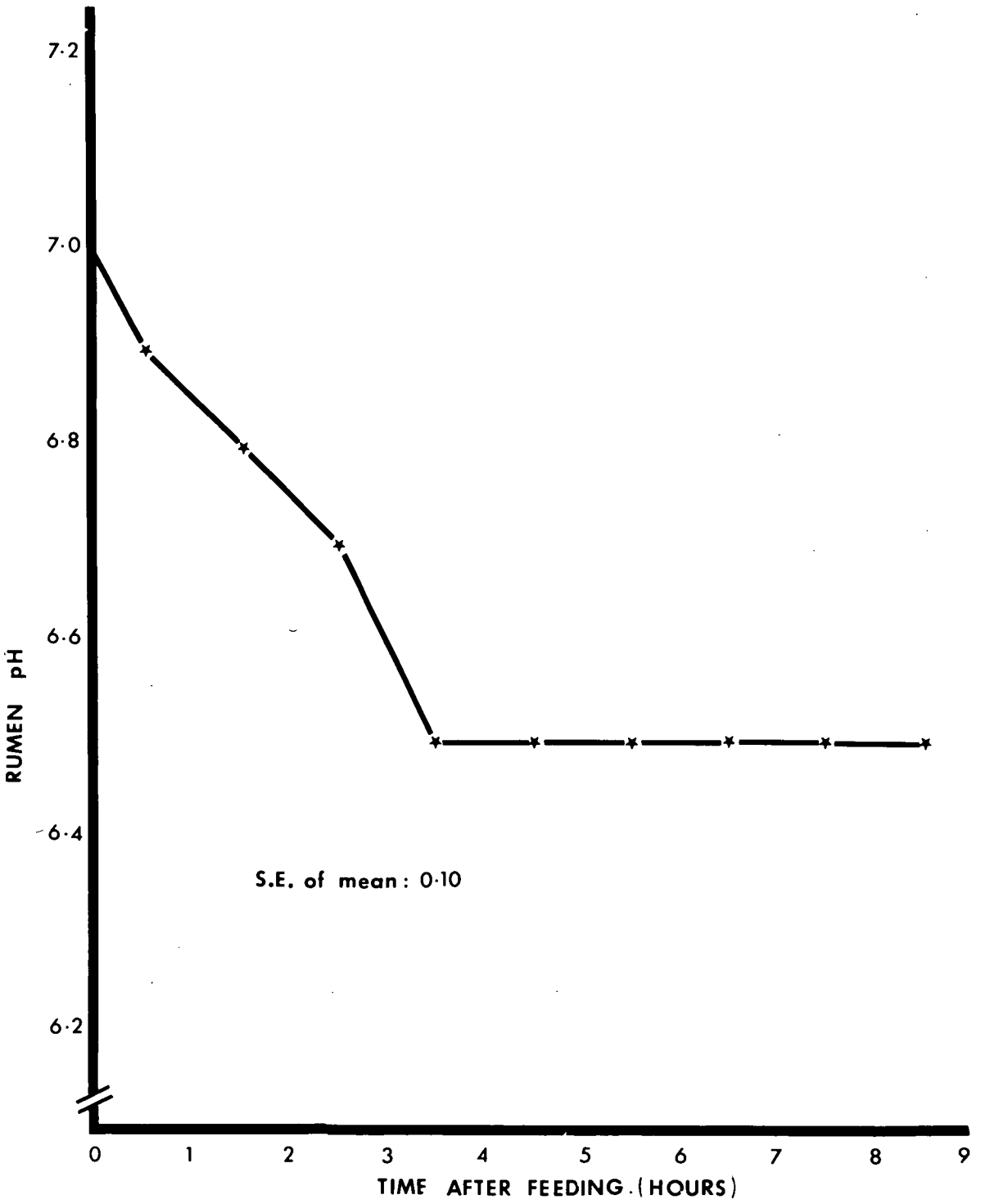

7·2
7·0
6·8
6·6
6·4
6·2
RUMEN pH
S.E. of mean : 0·10
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING. (HOURS)

Fig. 8 The effect of dietary molybdenum on rumen redox potentials in sheep fed four levels of molybdenum, namely 40 ppm, 240 ppm, 720 ppm and 1440 ppm (means of four sheep).

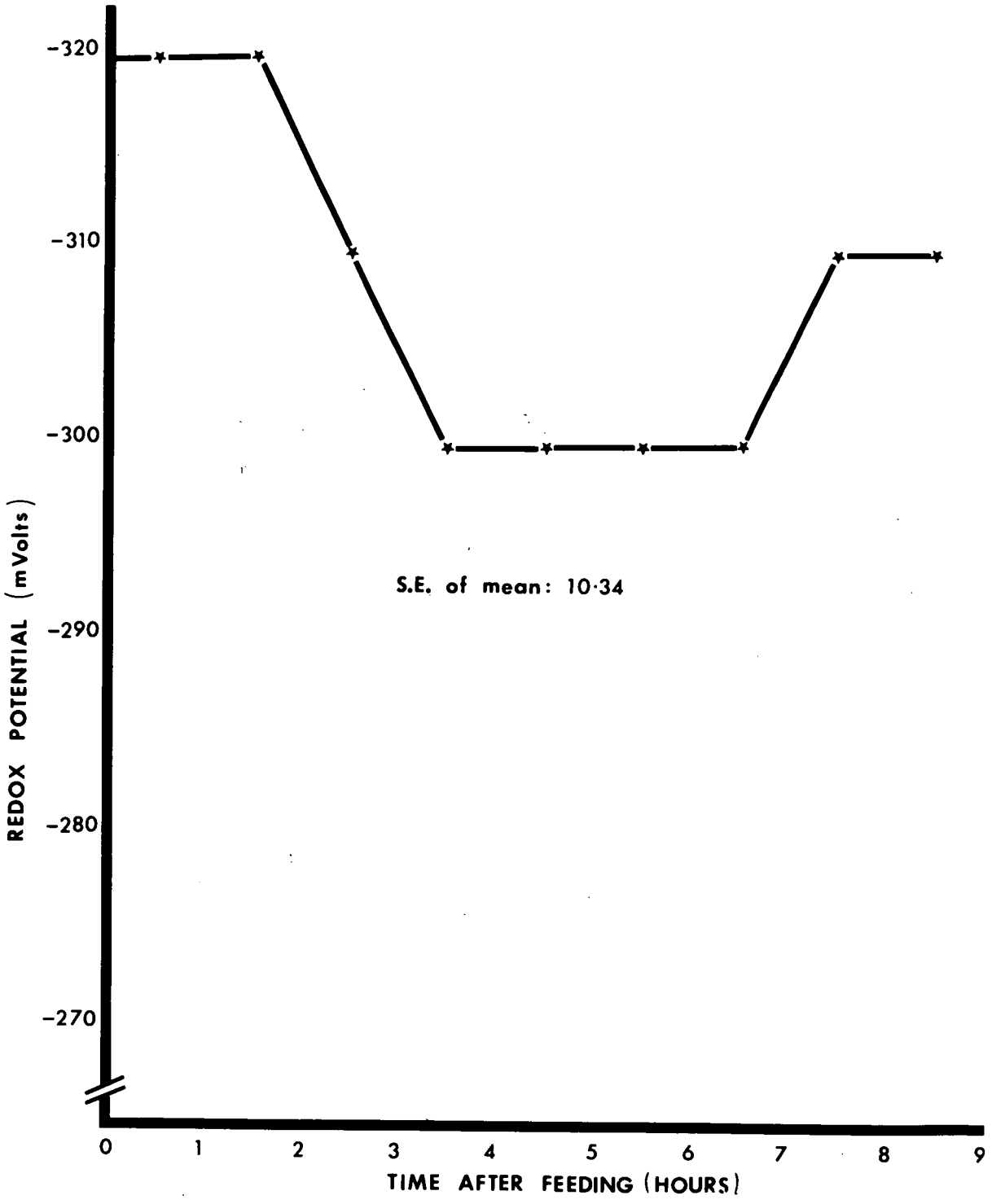
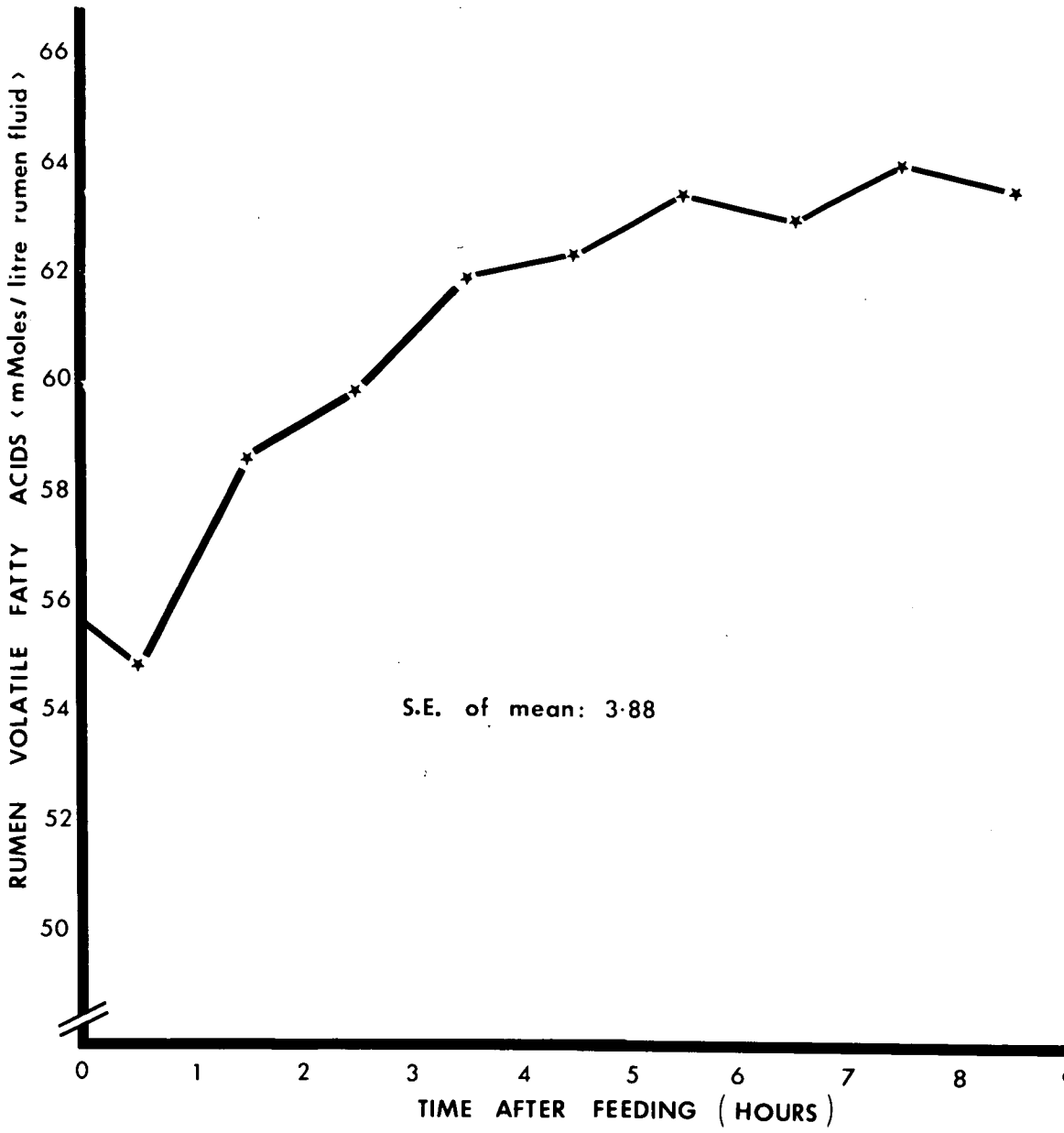

-320
-310
-300
-290
-280
-270
REDOX POTENTIAL (mVolts)
S.E. of mean: 10·34
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

Fig. 9 The concentration of steam volatile fatty acids in the rumen of sheep fed four levels of molybdenum, namely 40 ppm, 240 ppm, 720 ppm and 1440 ppm (means of four sheep).


RUMEN VOLATILE FATTY ACIDS (mMoles / litre rumen fluid)
66
64
62
60
58
56
54
52
50
S.E. of mean: 3·88
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

There was no significant difference between treatments on the digestion rate of cotton thread, nor on the total numbers of rumen bacteria.

However the dietary molybdenum in all treatments significantly reduced the numbers of rumen sulphate-reducing bacteria from that of the basal 6.5×10^6 to 3.8×10^2 ($P < 0.01$) (see Table 15).

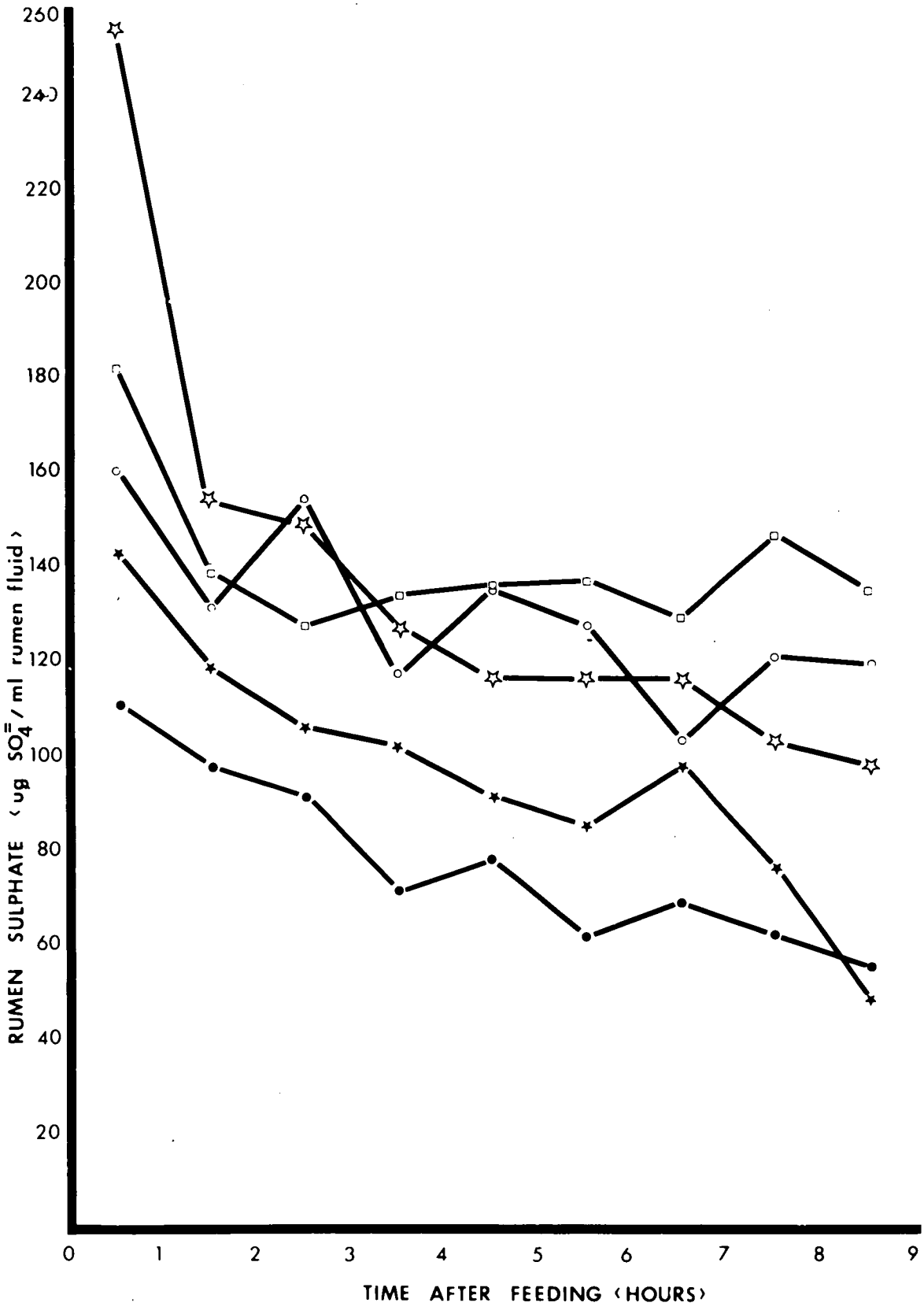
Table 15

The effect of dietary molybdenum on the numbers of rumen sulphate-reducing bacteria measured 8.5 hours after feeding (means of four sheep).

Treatment	Sulphate-reducing bacteria per ml of rumen fluid
basal	6.45×10^6
A	6.0×10^2
B	4.6×10^2
C	3.7×10^2
D	0.95×10^2

Rumen sulphate levels were measured (see Figure 10). A significant difference ($P < 0.05$) occurred between dietary treatments indicating that as the amount of molybdenum increased in the diet, the level of sulphate in the rumen also increased. The increase in sulphate levels was presumably due to the inhibition of the reductive pathway by the higher levels of dietary molybdenum.

Fig. 10 Rumen sulphate concentrations in the rumen of sheep fed diets containing molybdenum at the following concentrations: <0.7 ppm ✱——✱; 40 ppm •——•; 240 ppm ×——×; 720 ppm ○——○; 1440 ppm □——□ (means of four sheep).


260
240
220
200
180
160
140
120
100
80
60
40
20
RUMEN SULPHATE (ug $SO_4^=$ / ml rumen fluid)
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

Experiment B(3)

In experiment B(2), although the addition of dietary molybdenum inhibited the rumen sulphide production substantially below that of the basal level, never at any stage did the rumen sulphide level reach zero. This could have been due to a number of factors such as the level of sulphur in the basal diet, the numbers of sulphate-reducing bacteria present in the rumen when a supplement of 1.5 per cent sulphate was fed, or due to protein catabolism in the rumen. Experiment B(3) was designed to determine the effects of diets with and without added sodium sulphate; superimposed on these were the effects of diets with and without added molybdenum. The sheep were offered a basal ration of oaten chaff, molasses, urea and trace-element mix similar to that given in Table 7, plus molybdate and sulphate as set out in Table 16. Each sheep was offered 600 gm dry matter per day. The experimental design was a 4 x 4 Latin square.

Results

The same parameters were measured as in experiment B(2). The levels of rumen sulphide were taken as evidence of the extent of sulphate reduction when the different diets were fed (see Figure 11).

In the absence of sulphate, the level of rumen sulphide was reduced to zero irrespective of molybdenum treatment. When sodium sulphate was added the level of rumen sulphide increased substantially.

However, addition of molybdenum reduced the rumen sulphide level below that of sulphate alone, but this level never approached zero, indicating that the presence of molybdenum in diets containing supplementary sulphate, inhibits sulphate reduction.

Table 16

Composition of experimental rations (% D.M.) used in experiment B(3).

Component	Diets			
	A	B	C	D
Na_2SO_4	0	1.5	0	1.5
$Na_2MoO_4.2H_2O$	0	0.15	0.15	0
Mo-content (ppm)	<0.7	1500	1500	<0.7
Daily Mo intake (mg)	<0.9	900	900	<0.9
Daily S intake (gm)	0.08	2.0	0.08	2.0

The rumen sulphate levels (see Figure 12) indicated that diets without sodium sulphate (whether molybdenum was added or not) contained a small amount of total sulphate sulphur which was presumably derived from the recycling of sulphate. When sulphate was added to the diet the levels of rumen sulphate sulphur increased significantly ($P < 0.05$), but no significant difference in sulphate levels was apparent between diets containing sulphate with and without added molybdenum.

Fig. 11 The effect of dietary molybdate and inorganic sulphate on the production of sulphide in the rumen of sheep fed the following diets: minus sulphate, minus molybdenum ● —— ●; minus sulphate, plus molybdenum × —— ×; plus sulphate, minus molybdenum ○ —— ○; plus sulphate, plus molybdenum ▫ —— ▫ (means of four sheep).

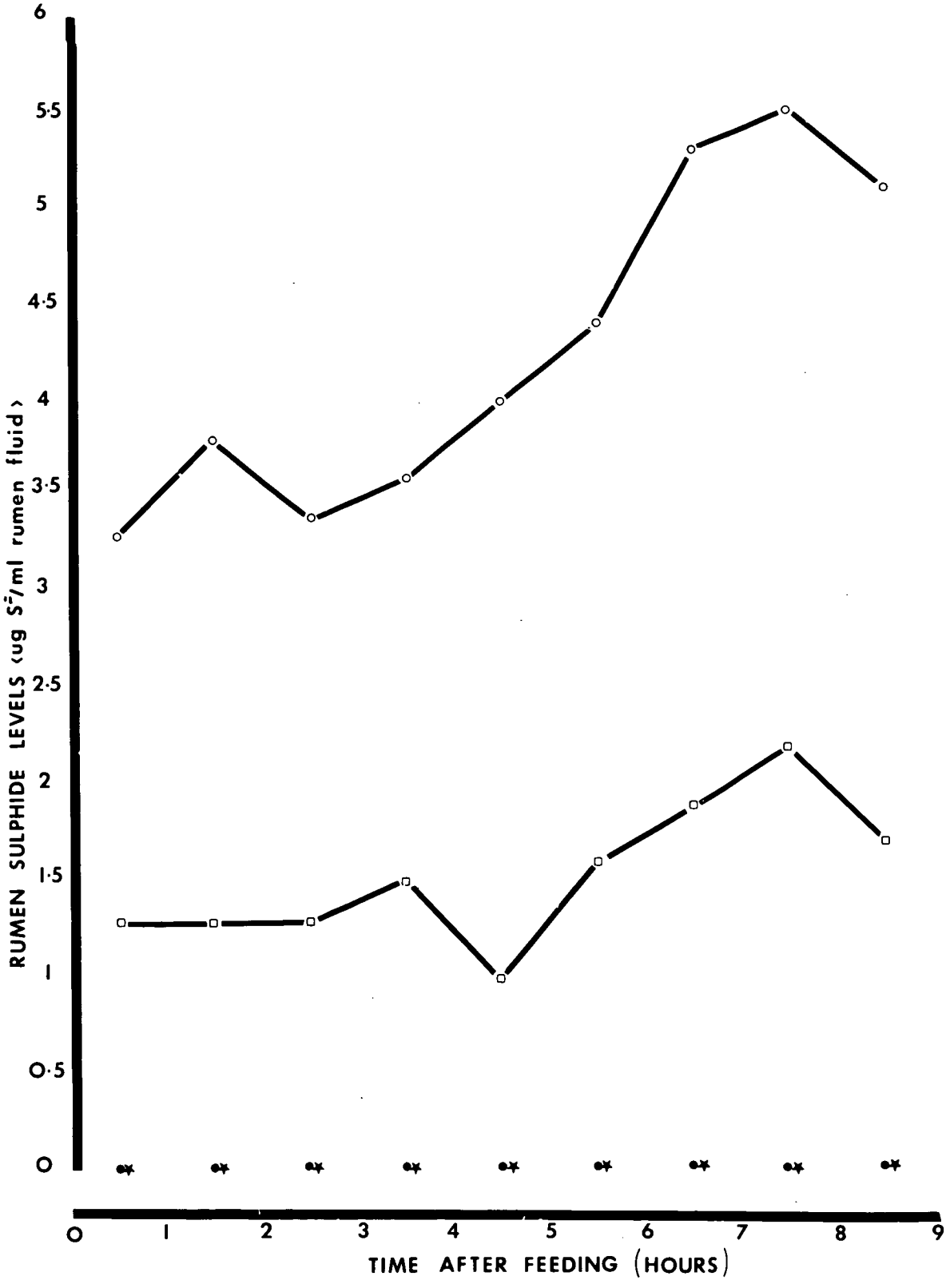
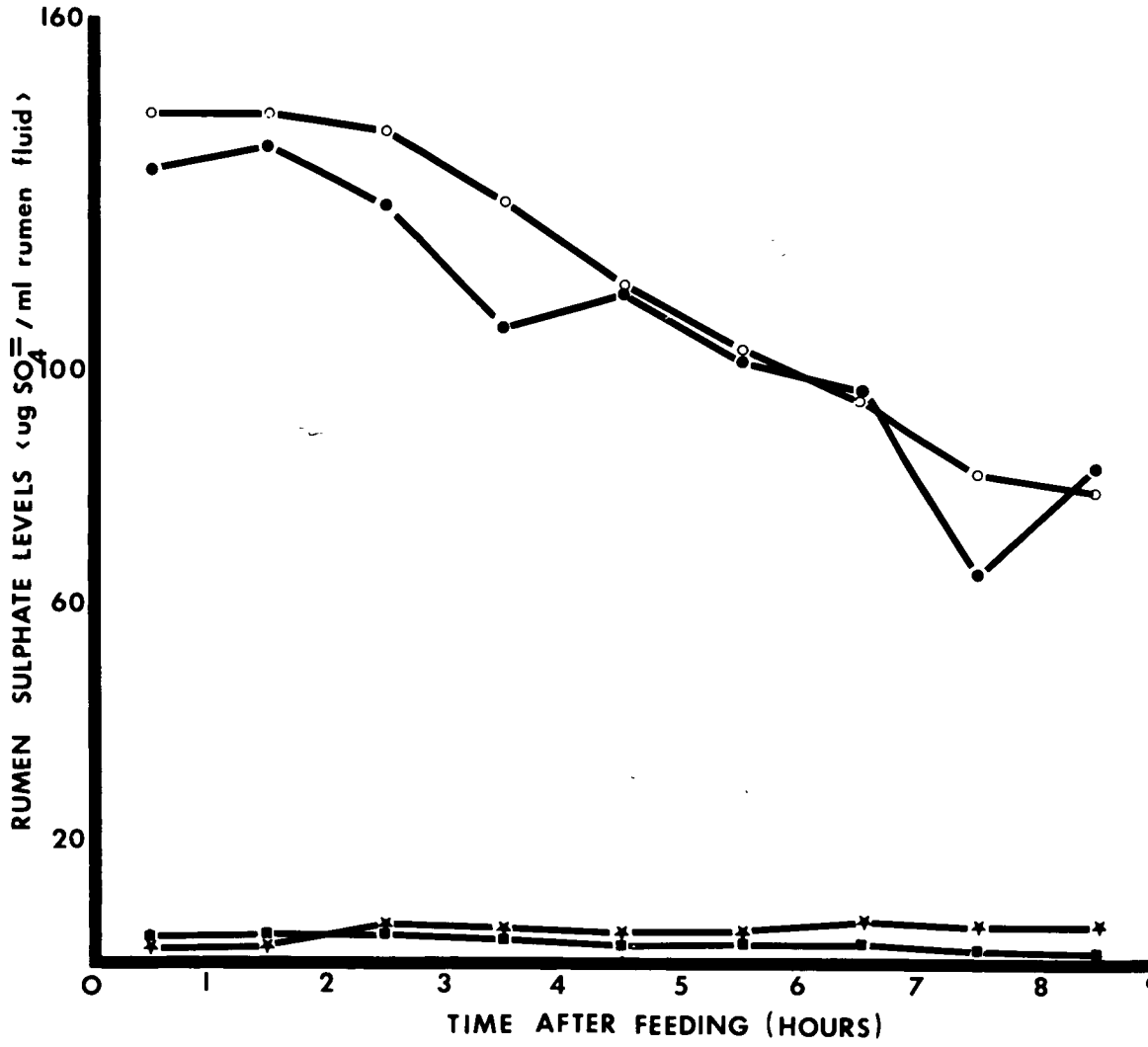

RUMEN SULPHIDE LEVELS (ug S⁼/ml rumen fluid)
6
5·5
5
4·5
4
3·5
3
2·5
2
1·5
1
0·5
0
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

Fig. 12 The effect of dietary molybdate and inorganic sulphate on the rumen sulphate concentration in sheep fed the following diets: minus sulphate, minus molybdenum ■ —— ■; minus sulphate, plus molybdenum × —— ×; plus sulphate, minus molybdenum, minus molybdenum ○ —— ○; plus sulphate, plus molybdenum ● —— ●; (means of four sheep).


160
100
60
20
RUMEN SULPHATE LEVELS (ug $SO_4^{=}$ / ml rumen fluid)
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

The concentration of molybdenum in the rumen was positively correlated to dietary molybdenum levels and the variation of rumen molybdenum concentration with time for each treatment is shown in Figure 13. As in experiment B(2) these levels were relatively constant over the 8.5 hour sampling period although a decrease in concentration with time would normally have been expected.

Differences in feed consumption occurred between different diets, but these differences were not significant. The lowest intakes occurred in the diet containing molybdenum but without sulphate.

There was no significant effect due to dietary treatments on rumen pH, rumen redox potentials or steam volatile fatty acid concentrations (see Figures 14, 15 and 16).

The digestion times for cotton thread bundles, the total numbers of rumen bacteria and the numbers of sulphate-reducing bacteria during each treatment are presented in Table 17.

The addition of sulphate to both the diets C and D caused a highly significant increase ($P < 0.001$) in the rate of cotton thread digestion (diets C and D versus A and B). There was no significant difference between either diets A and B or C and D indicating that the molybdenum supplement in the diet had no effect on cellulose digestion. There was no effect on the total number of rumen bacteria due to either sulphur or molybdenum treatment, but the addition of sulphur without molybdenum (diet C) resulted in a significant increase ($P < 0.001$) in the numbers of sulphate-reducing bacteria. In the diets without added sulphur (diets A and B) the effect of molybdenum was

Fig. 13 Rumen molybdenum concentration in sheep fed the following diets: minus sulphate, minus molybdenum ✾——✾; minus sulphate, plus molybdenum■——■; plus sulphate, minus molybdenum ○——○; plus sulphate, plus molybdenum●——●(means of four sheep).

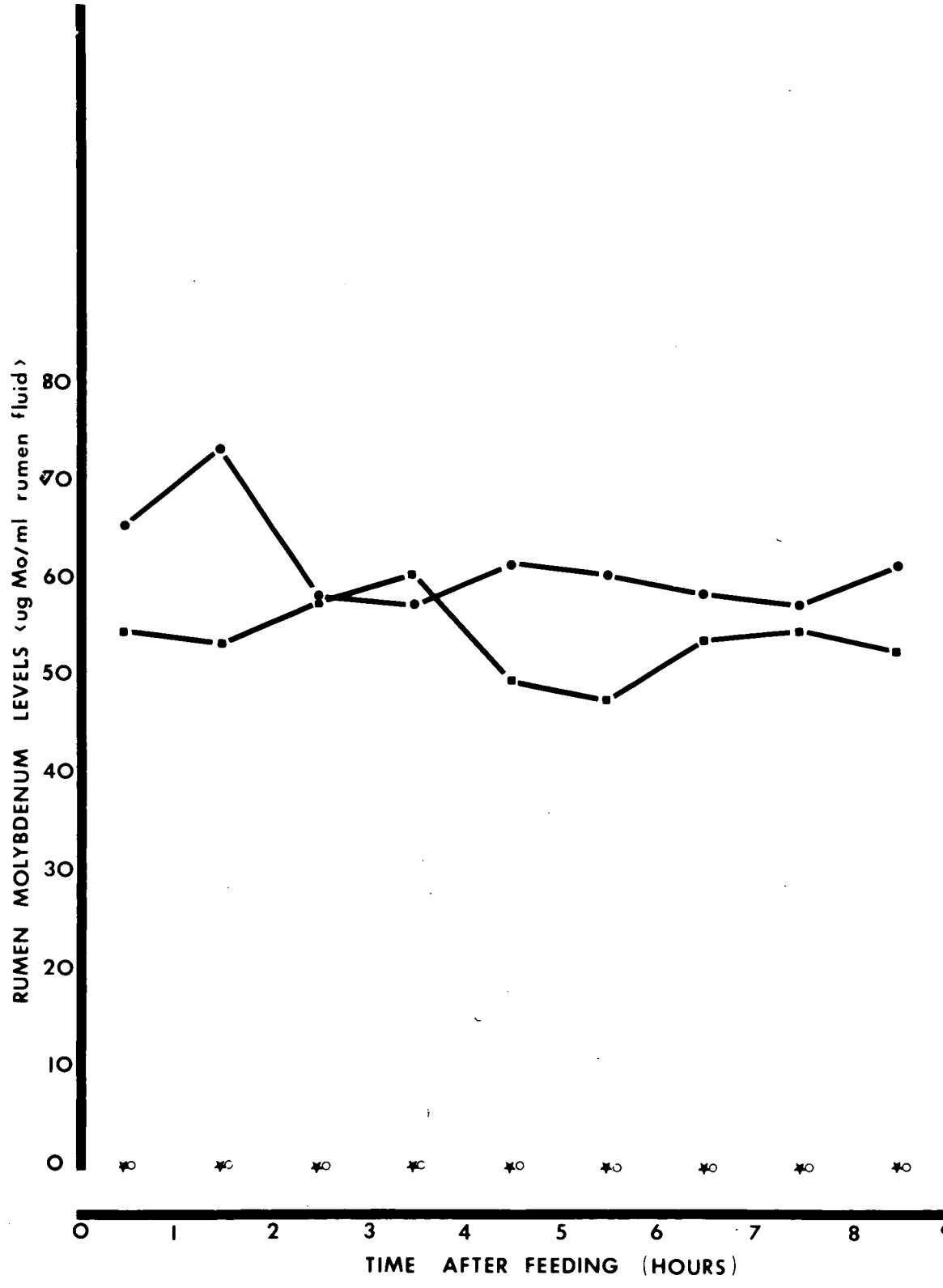

RUMEN MOLYBDENUM LEVELS (ug Mo/ml rumen fluid)
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

Fig. 14 The effects of dietary molybdate and inorganic sulphate on rumen pH in sheep fed the following diets: minus sulphate, minus molybdenum; minus sulphate, plus molybdenum; plus sulphate, minus molybdenum; plus sulphate, plus molybdenum (means of four sheep).

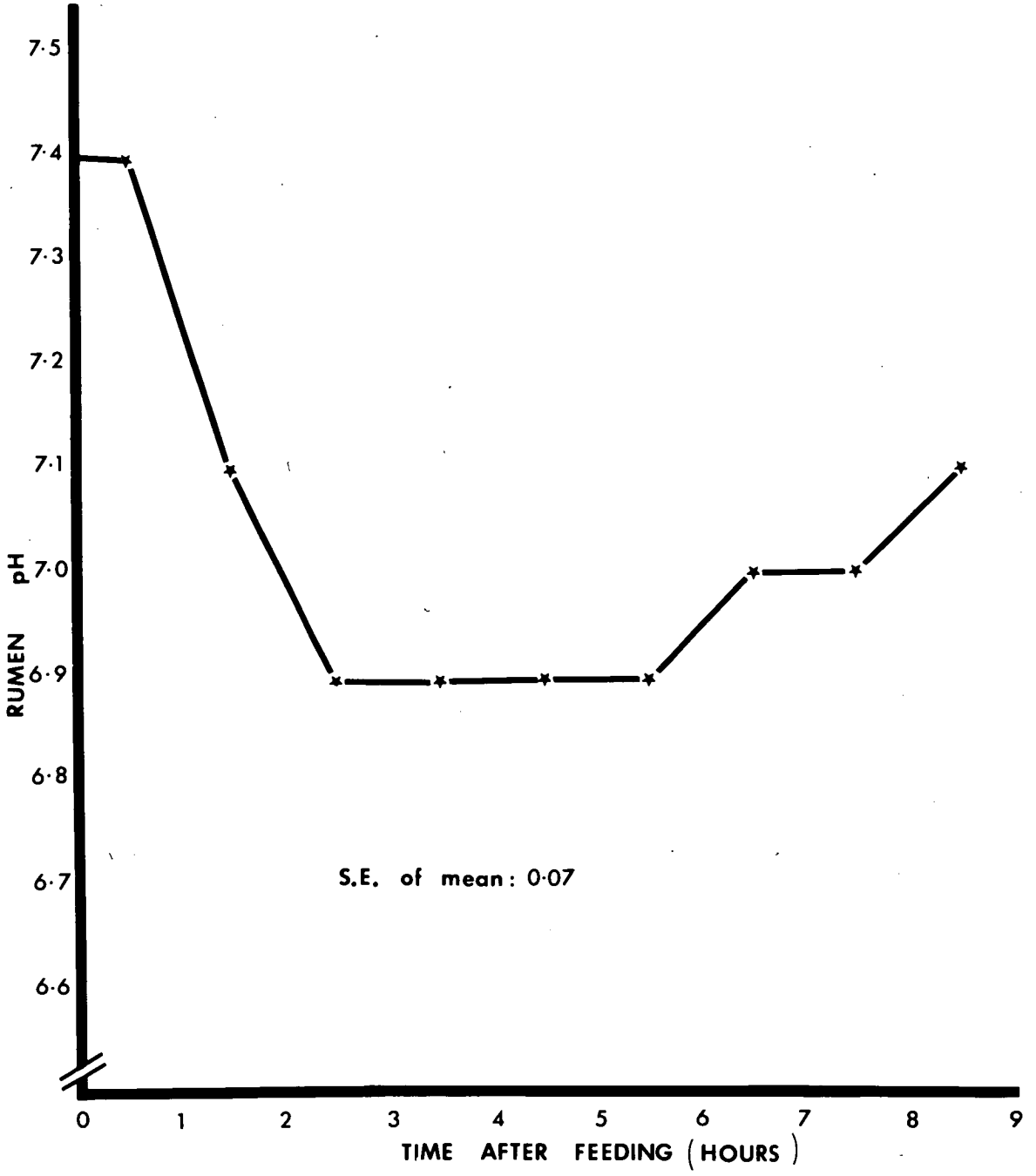

7·5
7·4
7·3
7·2
7·1
7·0
6·9
6·8
6·7
6·6
RUMEN pH
S.E. of mean: 0·07
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

Fig. 15 The effects of dietary molybdate and inorganic sulphate on rumen redox potentials in sheep fed the following diets: minus sulphate, minus molybdenum; minus sulphate, plus molybdenum; plus sulphate, minus molybdenum; plus sulphate, plus molybdenum (means of four sheep).

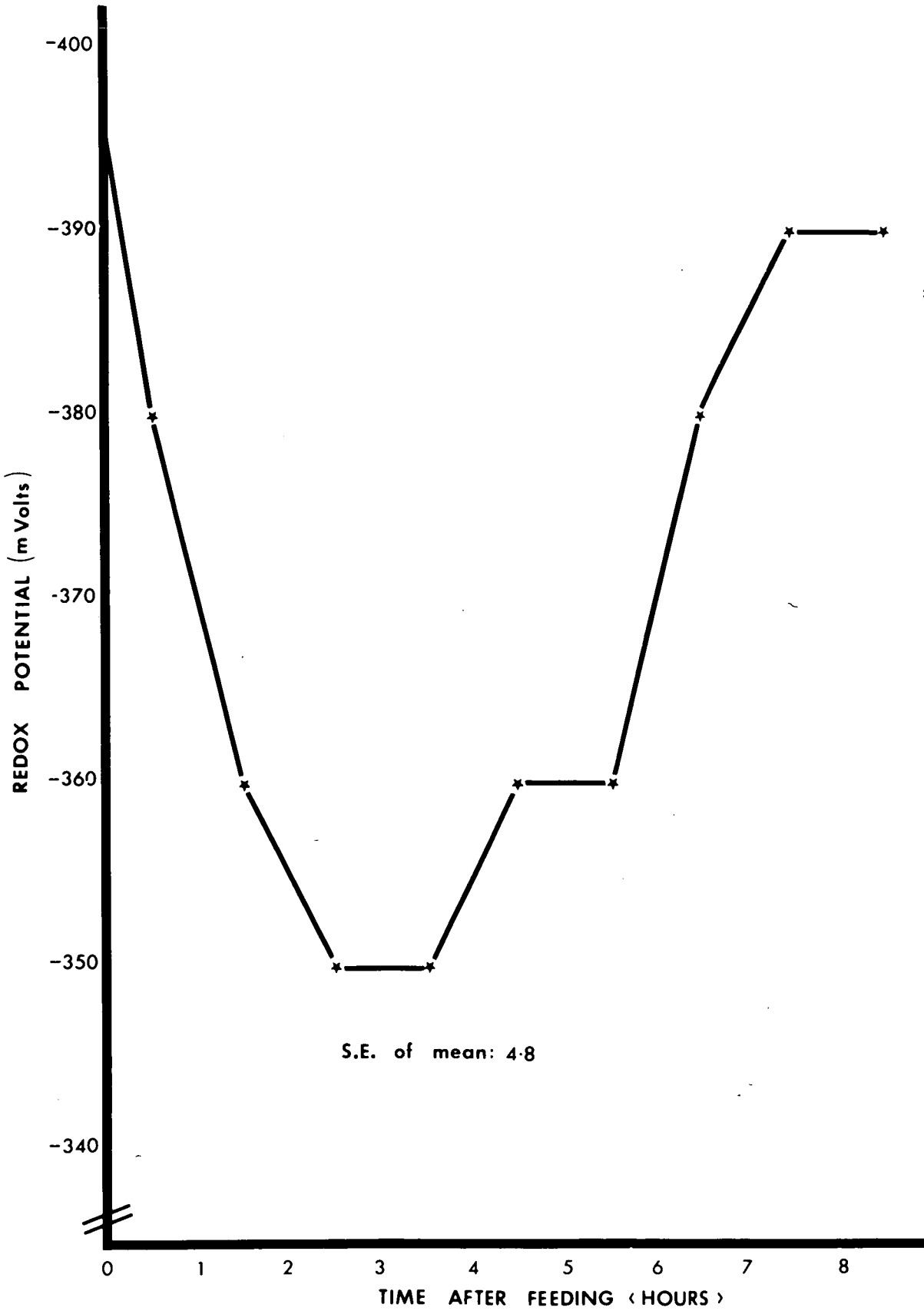
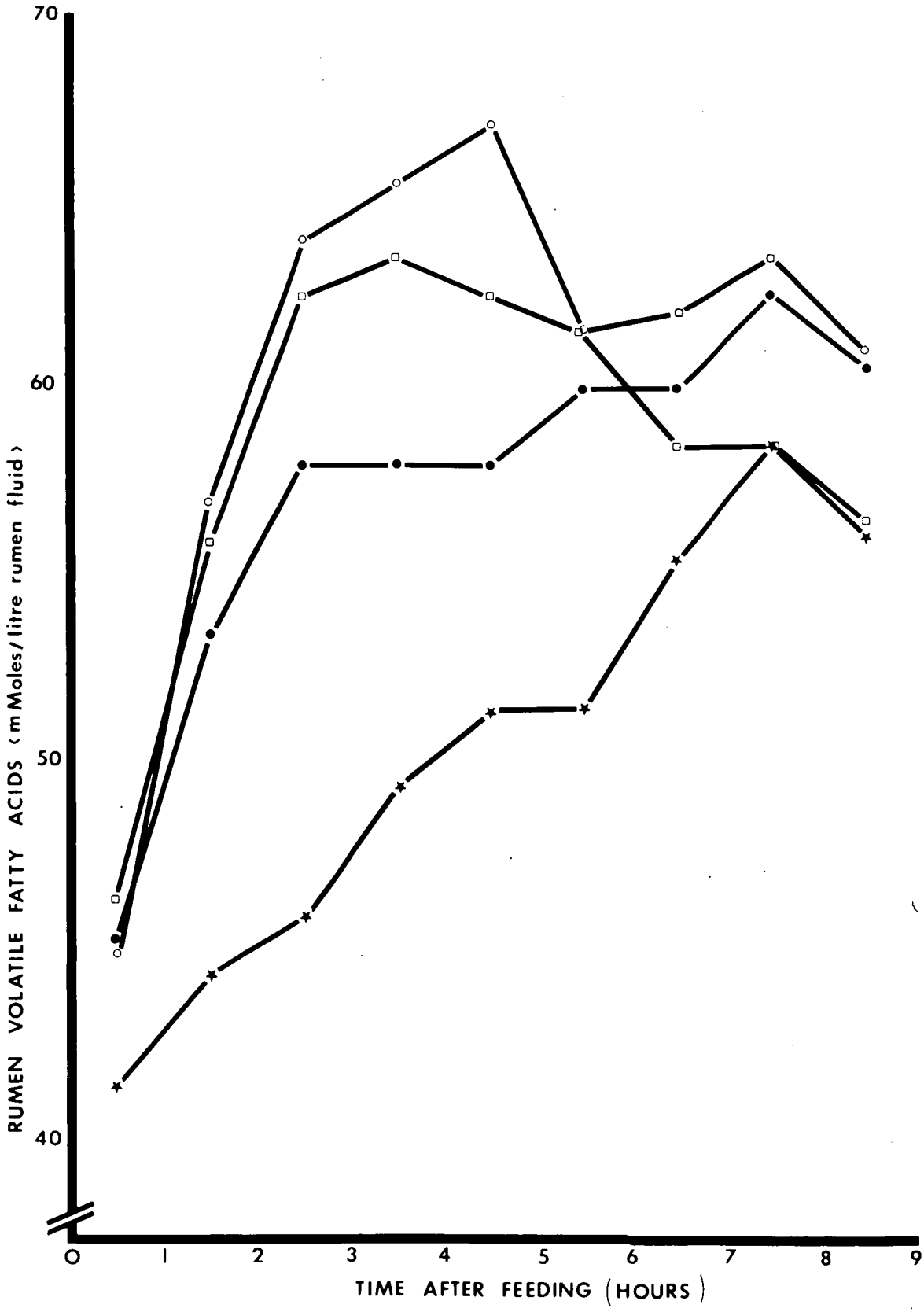

-400
-390
-380
-370
-360
-350
-340
REDOX POTENTIAL (m Volts)
S.E. of mean: 4·8
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING ‹ HOURS ›

Fig. 16 The effects of dietary molybdate and inorganic sulphate on the concentration of steam volatile fatty acids in the rumen of sheep fed the following diets: minus sulphate, minus molybdenum ●——●; minus sulphate, plus molybdenum ×——×; plus sulphate, plus molybdenum □——□; plus sulphate, minus molybdenum ○——○ (means of four sheep).


70
60
50
40
RUMEN VOLATILE FATTY ACIDS (m Moles/litre rumen fluid)
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

negligible, but in diets which contained a sulphate supplement, molybdenum caused a significant decrease in the sulphate-reducing bacteria numbers ($P < 0.05$).

Table 17

The effect of dietary molybdate and inorganic sulphate on the digestion of cotton thread bundles placed in the rumen, the numbers of total rumen bacteria and numbers of sulphate-reducing bacteria measured 8.5 hours after feeding (means of four sheep).

Treatment	Hours taken for 50% weight loss of cotton thread	Total bacteria per ml of rumen fluid	Sulphate-reducing bacteria per ml rumen fluid
-S-Mo (A)	68	17.7×10^{10}	8.0×10^{2}
-S+Mo (B)	67	28.5×10^{10}	3.0×10^{2}
+S-Mo (C)	38.8	14.4×10^{10}	1.12×10^{6}
+S+Mo (D)	39.3	11.6×10^{10}	1.3×10^{2}

The molybdenum, nitrogen and sulphur balance figures are presented in Table 18.

Table 18

The effects of dietary molybdate and inorganic sulphate on the daily molybdenum, nitrogen and sulphur balance figures (means of four sheep).

Diets	Mo balance (mg)	N balance (g)	S balance (g)
-S-Mo (A)	0	0.25	0.01
-S+Mo (B)	0.43	0.03	0.01
+S-Mo (C)	0	0.21	0.04
+S+Mo (D)	0.26	0.19	0.03

The addition of molybdenum (diets B and D) caused a significant increase ($P < 0.01$) in the molybdenum balance when compared to the diets without molybdenum (diets A and C), but the addition of sulphur and molybdenum (diet D) caused a significant decrease ($P < 0.05$) in molybdenum balance compared to the diet with molybdenum alone.

Nitrogen balance figures in the diet with molybdenum but without added sulphate (diet B) were significantly lower ($P < 0.001$) than when diets A, C and D were fed. However, there was no significant difference between diets A, C and D.

There was a significant increase ($P < 0.05$) in sulphur balance with added sulphate (diets C and D) when compared to diets without sulphate (diets A and B). There was no significant effect of molybdenum on sulphur balance.

Experiment B(4)

Following the results of epxeriment B(2) in which it was established that a level of 40 ppm molybdenum stimulated inorganic sulphate reduction in the rumen and 240, 720 and 1440 ppm inhibited inorganic sulphate reduction as measured by rumen sulphide production, experiment B(4) was designed to determine the critical level of molybdenum stimulation, and also a dose response curve by using a further six levels of dietary molybdenum ranging in concentration from 20 ppm to 160 ppm.

The basal ration of oaten chaff, molasses, urea, trace element mix and sodium sulphate was similar to that in Table 7. In addition, six levels of dietary molybdenum were fed (see Table 19). The experimental periods were divided in a way similar to that described in section B(1).

Table 19

Composition of experimental diets used in experiment B(4).

Diets	Component			
	$Na_2MoO_4.2H_2O$ (% D.M.)	Mo content (ppm)	Daily Mo intake (mg)	Daily S intake (g)
basal	0	<0.7	<0.9	2.8
A	2×10^{-3}	20	12	2.8
B	4.8×10^{-3}	48	29	2.8
C	7.5×10^{-3}	76	45.6	2.8
D	1×10^{-2}	104	62.4	2.8
E	1.3×10^{-2}	132	79.2	2.8
F	1.6×10^{-2}	160	96	2.8

Results

The same parameters were meased in experiment B(4) as in experiments B(2) and B(3). The effects of varied dietary levels of molybdenum on rumen sulphide concentration are shown in Figure 17. The control level was that established when the sheep were fed the basal ration without molybdenum.

There was a varied effect ($P < 0.01$) of molybdenum on rumen sulphide concentration. A dietary level of 20 ppm and 48 ppm stimulated sulphide production above that of the control whereas higher dietary levels (76, 104, 132 and 160 ppm) depressed rumen sulphide production. The levels of 132 and 160 ppm reduced the level of sulphide production to almost that of 240, 720 and 1440 ppm (see Figure 5).

A combination of results from experiments B(2) and B(4) (see Figure 18) shows that maximum stimulation of sulphide production was obtained with 40 ppm dietary molybdenum and that inhibition first occurred with 76 ppm molybdenum. While Figure 18 represents the rumen sulphide production at T30, a similar response occurred at all sampling times of the experiment. Levels between less than 0.7 ppm (basal ration) and 48 ppm Mo were all stimulatory and levels of 76 ppm molybdenum and greater were all inhibitory, reaching almost a plateau at 132 ppm to 1440 ppm molybdenum.

Rumen sulphate concentrations were measured (see Figure 19) to determine the effects of dietary molybdenum treatment on these sulphate levels. A statistical analysis on the effect of varying

Fig. 17 Sulphide concentration in the rumen of sheep fed diets containing molybdenum at the following concentrations: <0.7 ppm ●——●; 20 ppm × ——×; 48 ppm ● —— ●; 76 ppm ○——○; 104 ppm ✡ —— ✡; 132 ppm ▫ ——▫; 160 ppm ↘ —— ↘ (means of six sheep).

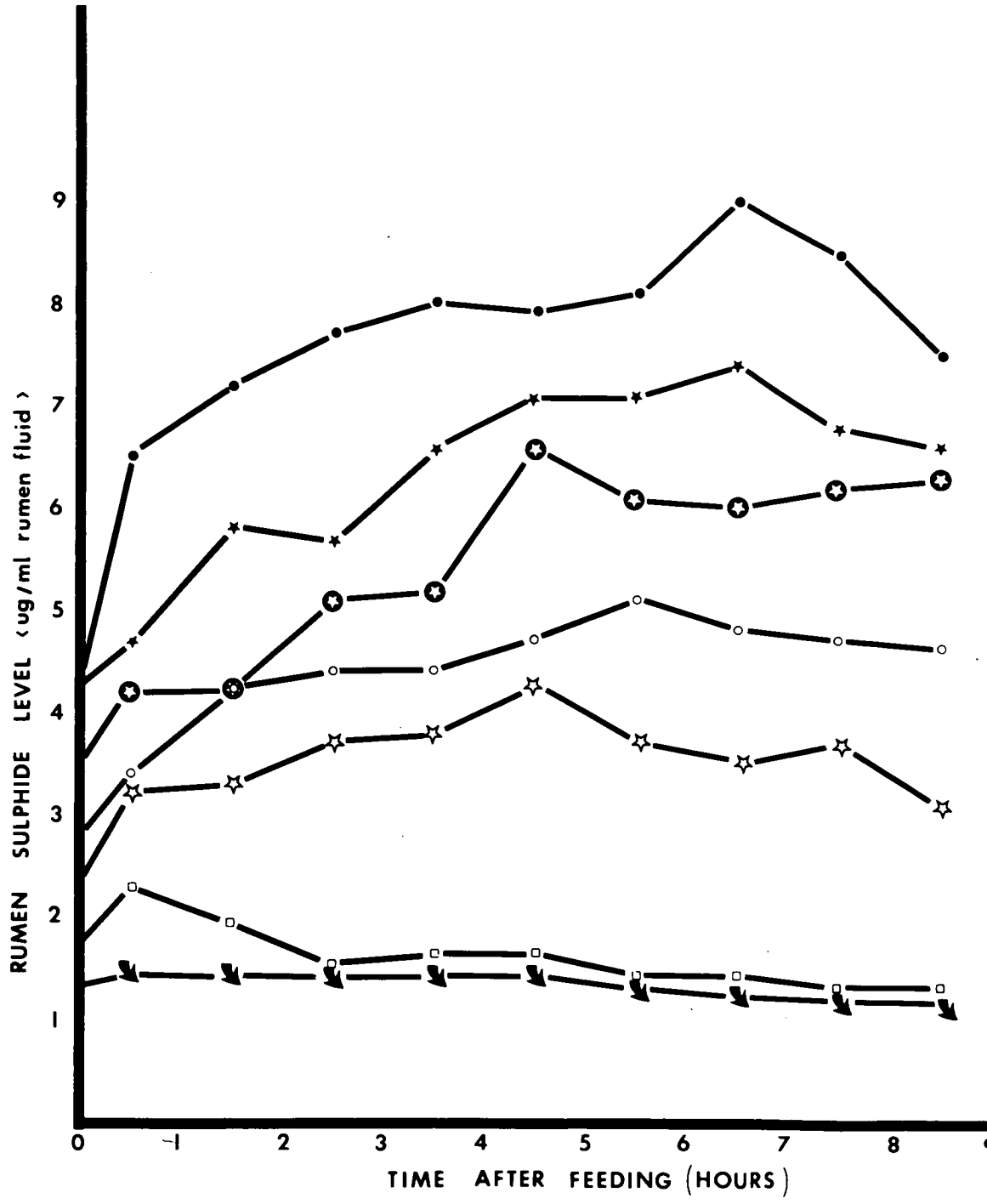

RUMEN SULPHIDE LEVEL (ug/ml rumen fluid)
9
8
7
6
5
4
3
2
1
0
-1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

Fig. 18 Sulphide concentration in the rumen of sheep fed diets containing ten different levels of molybdenum in experiments B(2) and B(4) at time T30.

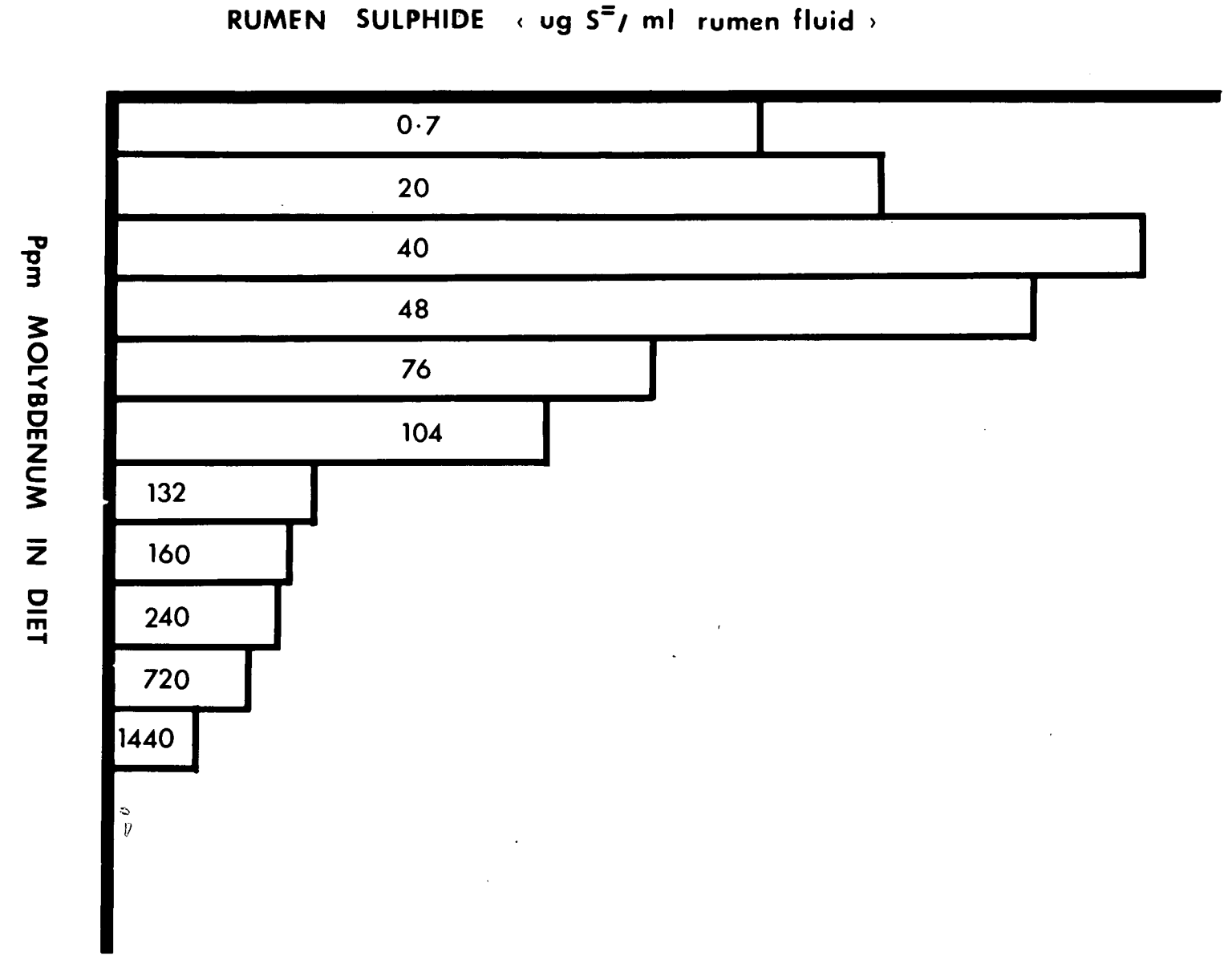
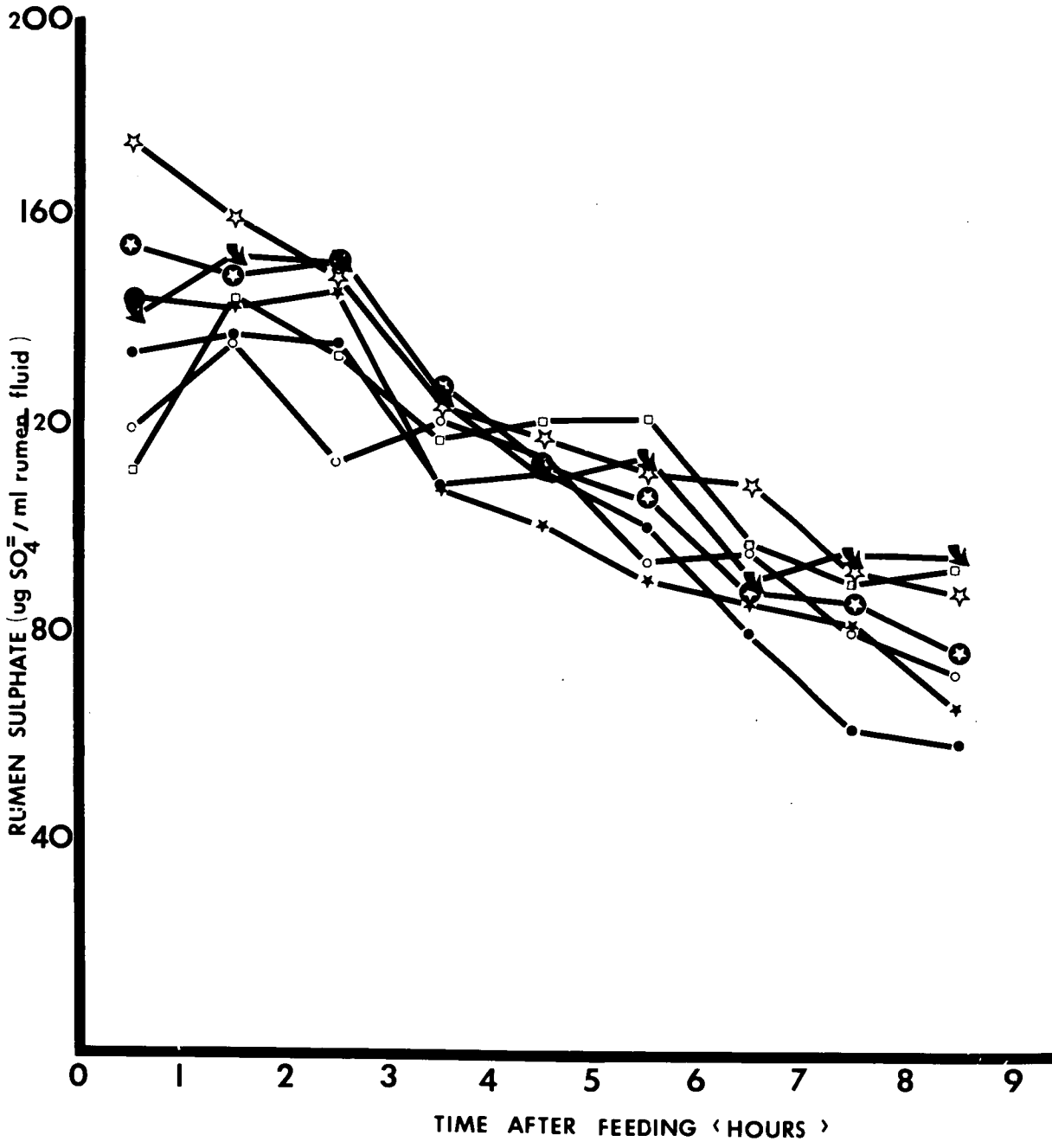

RUMEN SULPHIDE ‹ ug S= / ml rumen fluid ›
Ppm MOLYBDENUM IN DIET
0·7
20
40
48
76
104
132
160
240
720
1440

Fig. 19 Sulphate concentration in the rumen of sheep fed diets containing molybdenum at the following concentrations: <0.7 ppm ✡——✡; 20 ppm △——△; 48 ppm ●——●; 76 ppm ○——○; 104 ppm □——□; 132 ppm ↘——↘; 160 ppm ×——× (means of six sheep).


200
160
120
80
40
RUMEN SULPHATE (ug $SO_4^=$ / ml rumen fluid)
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING ‹ HOURS ›

dietary molybdenum on rumen sulphate concentrations showed no significant effect of molybdenum. A combination of rumen sulphate levels in experiments B(2) and B(4) with all the levels of dietary molybdenum (see Figure 20), again at T30, showed that the minimum rumen sulphate occurred with 40 ppm molybdenum, the level at which maximum rumen sulphide production occurred. The maximum level of sulphate concentration occurred with 1440 ppm molybdenum, the level at which minimum rumen sulphide occurred.

The concentration of molybdenum in the rumen (see Figure 21) varied with each treatment ($P < 0.001$) at all sampling times throughout the experiment, and the concentration in the rumen was positively correlated to dietary molybdenum concentration.

There was no effect of varied dietary levels of molybdenum on rumen pH, rumen redox potential or volatile fatty acid concentration in the rumen (see Figures 22, 23 and 24).

The digestion times for cotton thread bundles, the total numbers of rumen bacteria and the total numbers of sulphate-reducing bacteria during each dietary treatment are presented in Table 20.

There was no significant effect of varied dietary molybdenum on cellulose digestion in the rumen as measured by the cotton thread technique. The variation in the total number of rumen bacteria was not affected by dietary treatment although there was no significant effect between levels of molybdenum fed on the numbers of sulphate-reducing bacteria in the rumen where there was a significant reduction

Fig. 20 Sulphate concentration in the rumen of sheep fed diets containing ten different levels of molybdenum in experiments B(2) and B(4) at time T30.

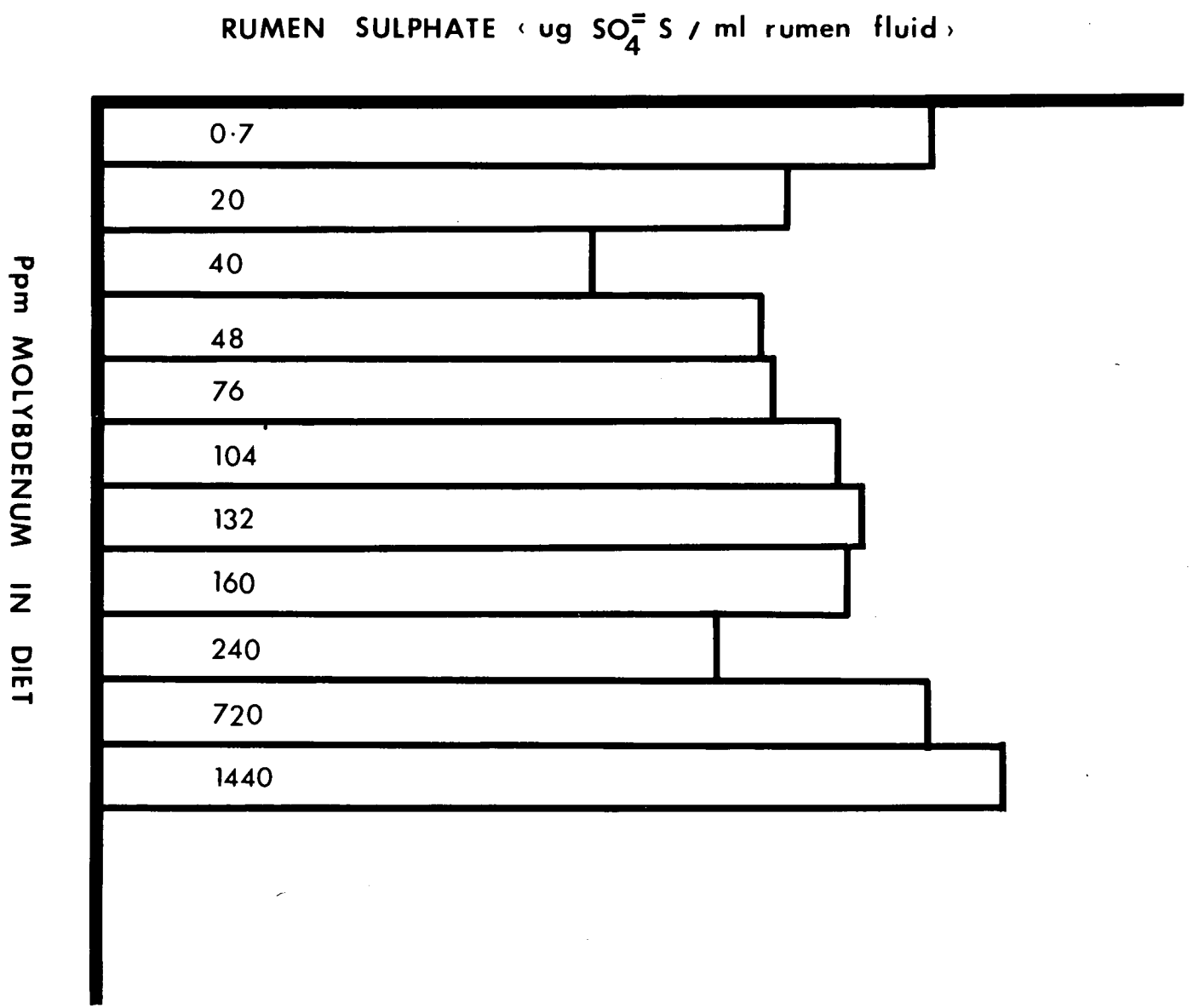

RUMEN SULPHATE ‹ ug $SO_4^=$ S / ml rumen fluid ›
Ppm MOLYBDENUM IN DIET
0·7
20
40
48
76
104
132
160
240
720
1440

Fig. 21 Molybdenum concentration in the rumen of sheep fed diets containing molybdenum at the following concentrations: 20 ppm ⊛—⊛; 48 ppm ×—×; 76 ppm •—•; 104 ppm ○—○; 132 ppm ✡—✡; 160 ppm □—□ (means of six sheep).

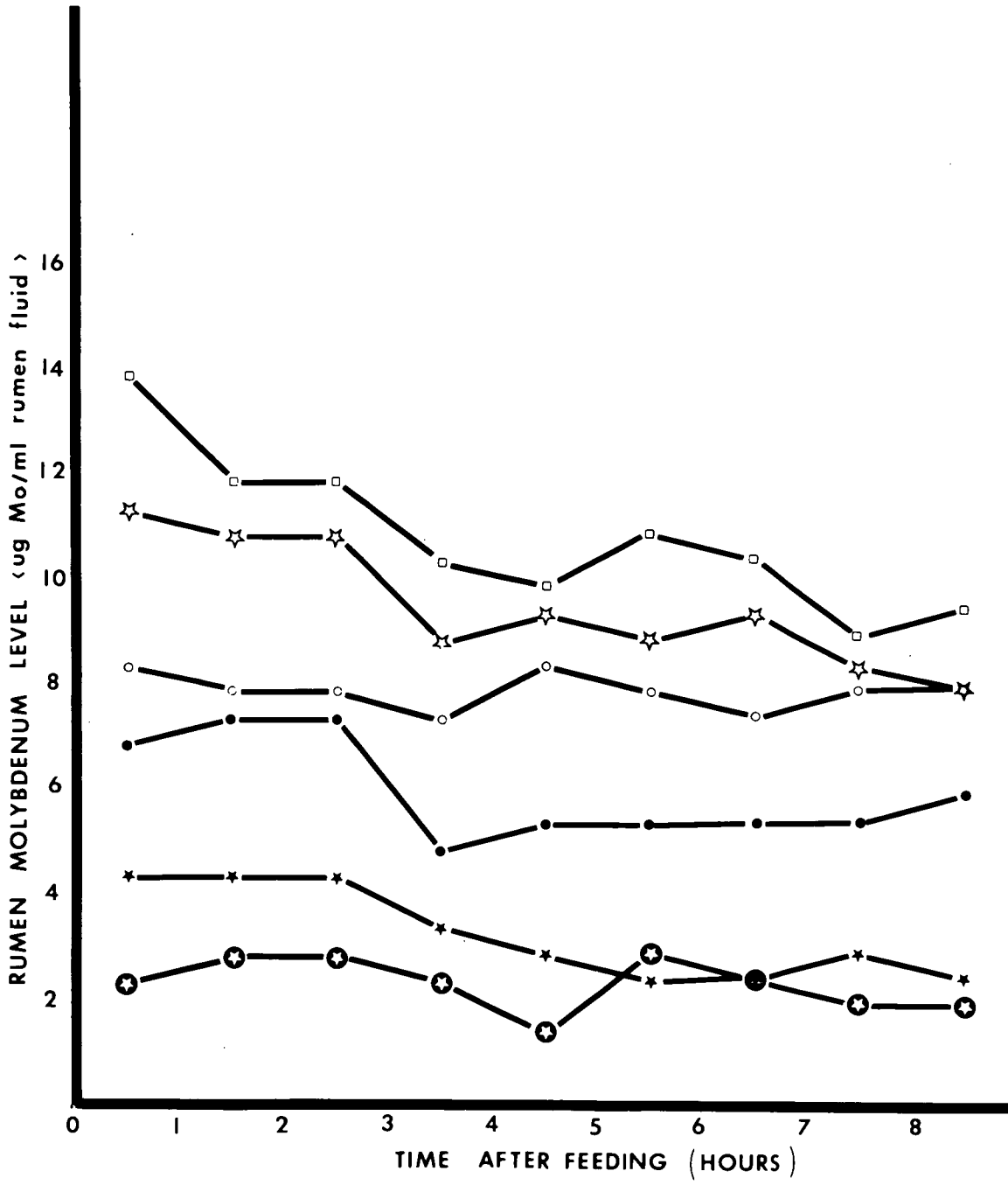

RUMEN MOLYBDENUM LEVEL (ug Mo/ml rumen fluid)
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

Fig. 22 The effect of dietary molybdenum on rumen pH in sheep fed six levels of molybdenum, namely 20 ppm, 48 ppm, 76 ppm, 104 ppm, 132 ppm and 160 ppm (means of six sheep).

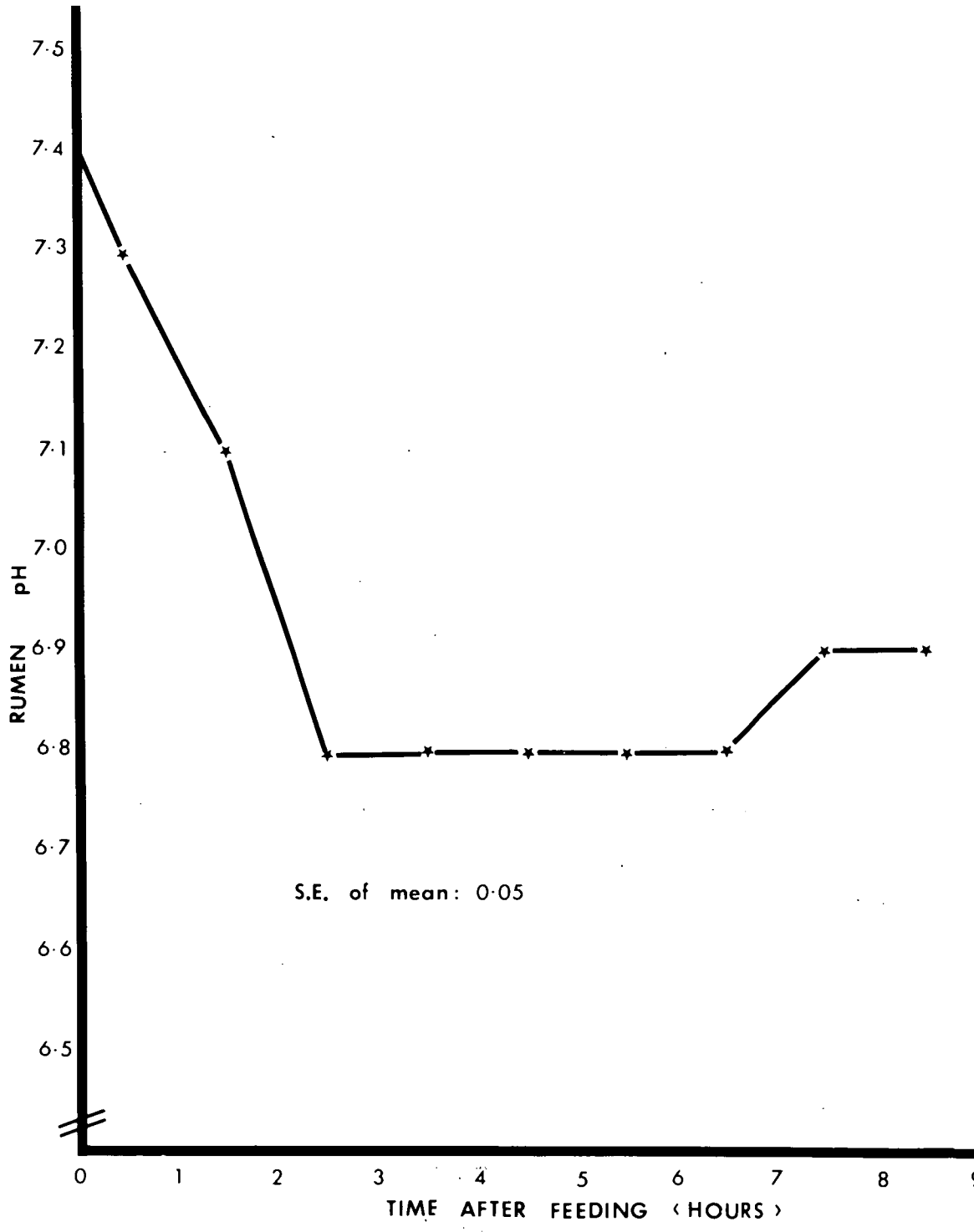

7·5
7·4
7·3
7·2
7·1
7·0
6·9
6·8
6·7
6·6
6·5
RUMEN pH
S.E. of mean: 0·05
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING ‹ HOURS ›

Fig. 23 The effect of dietary molybdenum on rumen redox potential in sheep fed six levels of molybdenum, namely 20 ppm, 48 ppm, 76 ppm, 104 ppm, 132 ppm and 160 ppm (means of six sheep).

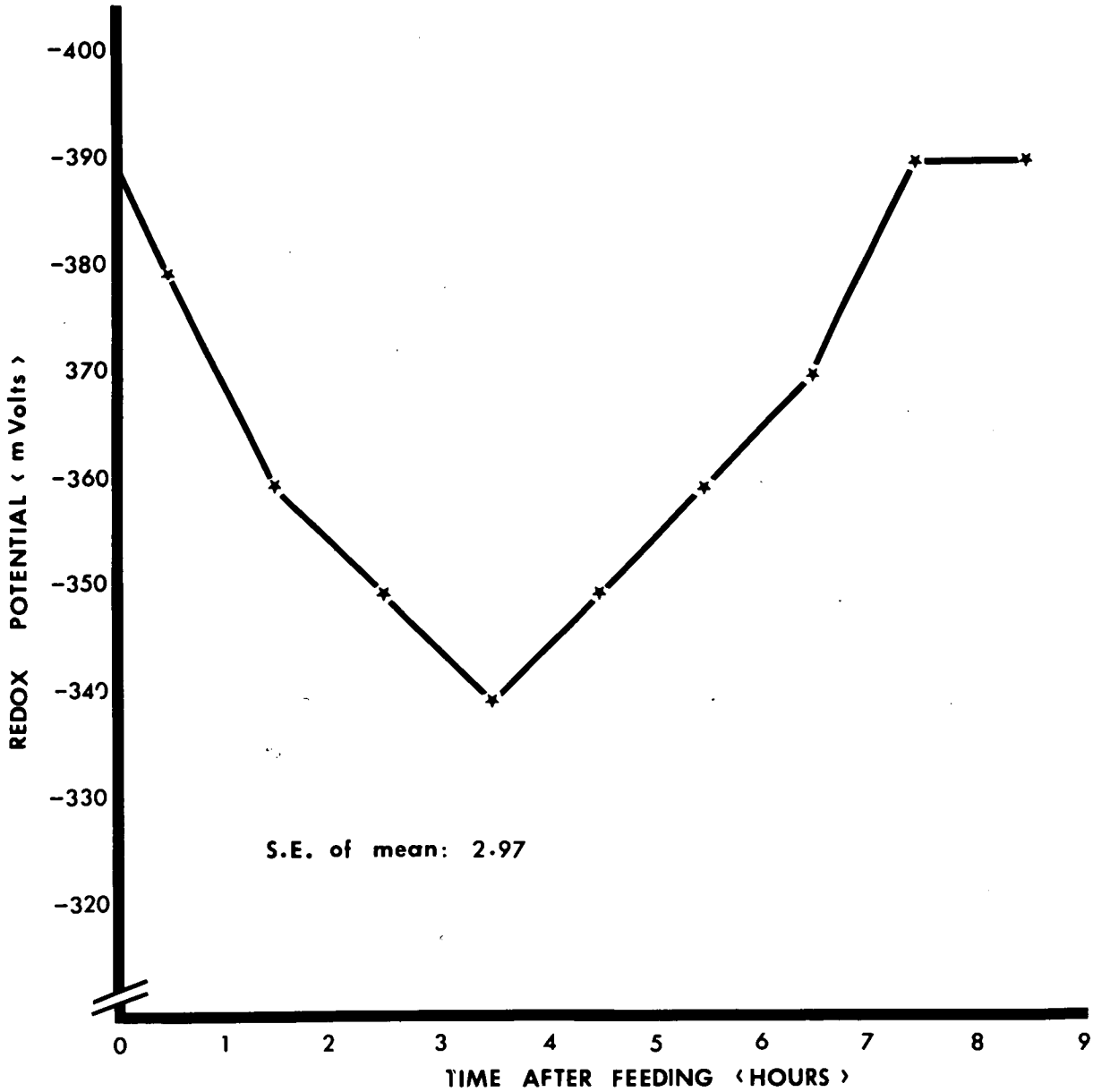
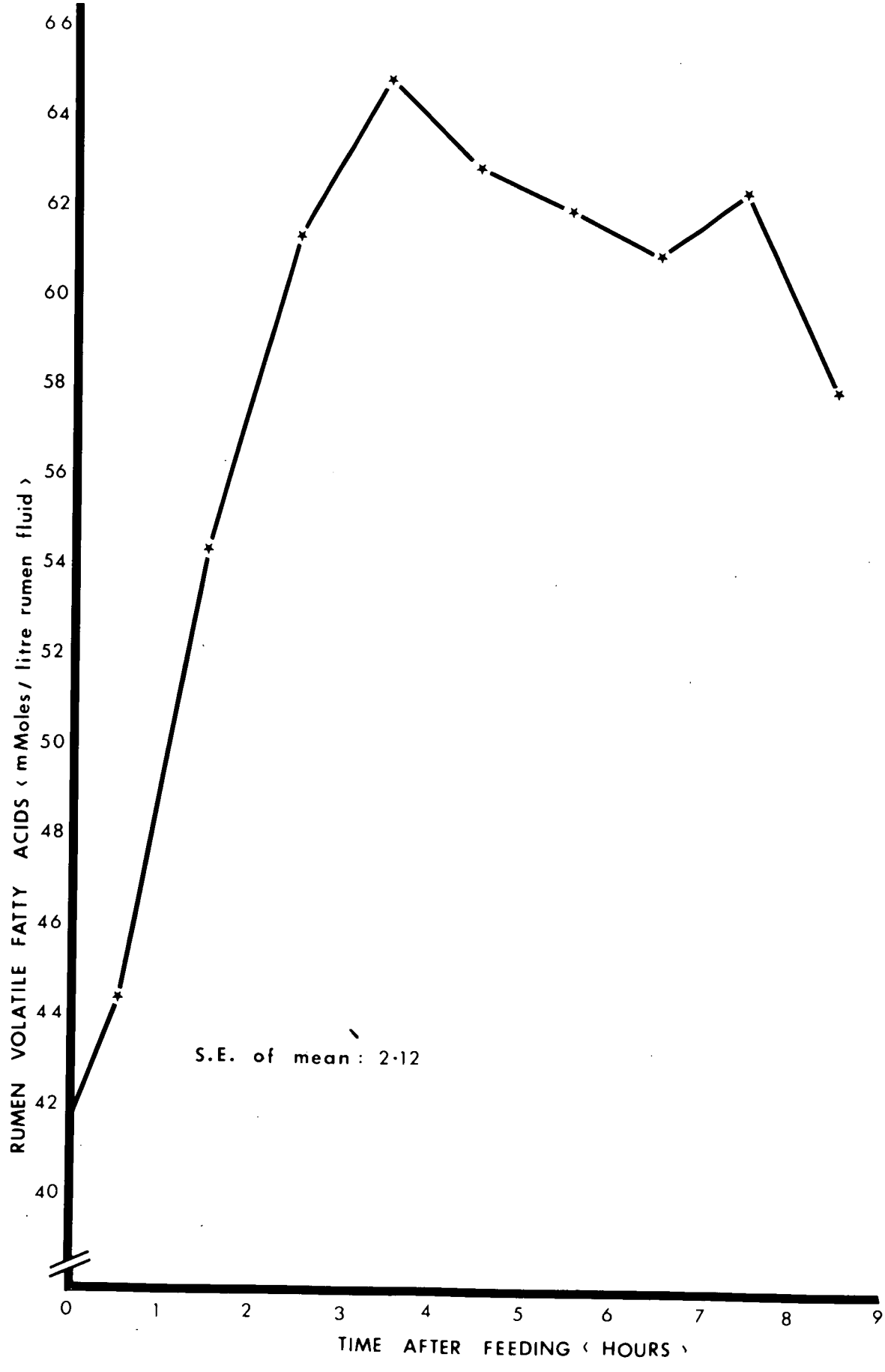

-400
-390
-380
370
-360
-350
-340
-330
-320
REDOX POTENTIAL (m Volts)
S.E. of mean: 2.97
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

Fig. 24 The effect of dietary molybdenum on rumen volatile fatty acids in sheep fed six levels of molybdenum, namely 20 ppm, 48 ppm, 76 ppm, 104 ppm, 132 ppm and 160 ppm (means of six sheep).


RUMEN VOLATILE FATTY ACIDS (mMoles / litre rumen fluid)
66
64
62
60
58
56
54
52
50
48
46
44
42
40
S.E. of mean : 2·12
0
1
2
3
4
5
6
7
8
9
TIME AFTER FEEDING (HOURS)

in sulphate-reducing bacteria ($P < 0.01$) between the basal ration and those rations containing the dietary molybdenum.

Table 20

The effect of dietary molybdenum on the digestion of cotton thread bundles placed in the rumen, the total number of rumen bacteria and numbers of sulphate-reducing bacteria measured 8.5 hours after feeding (means of six sheep).

Treatment	Hours taken for 50% weight loss of cotton thread	Bacteria per ml of rumen fluid	Sulphate-reducing bacteria per ml of rumen fluid
basal	39.8	9.4×10^{10}	2.9×10^{6}
A	39.5	1.5×10^{11}	1.8×10^{2}
B	39.2	1.3×10^{11}	3.1×10^{2}
C	39.5	1.1×10^{11}	1.2×10^{2}
D	38.3	1.1×10^{11}	8.8×10^{2}
E	38.0	1.3×10^{11}	1.6×10^{3}
F	38.3	1.5×10^{11}	8.8×10^{2}

The molybdenum, nitrogen and sulphur balance figures are presented in Table 21.

Table 21

The effect of dietary molybdenum on daily nitrogen, sulphur and molybdenum balance (means of six sheep).

Treatment	N-balance (g)	S-balance (g)	Mo-balance (mg)
basal	0.30	0.01	-
A	0.25	0.02	0.19
B	0.25	0.02	0.24
C	0.24	0.02	0.45
D	0.20	0.02	0.51
E	0.20	0.01	0.57
F	0.27	0.02	0.38

There was no significant difference due to treatment in the nitrogen and sulphur balance figures. However, as the level of molybdenum in the diet increased, the molybdenum balance figures also increased ($P < 0.05$), except in the diet containing 160 ppm molybdenum. In this treatment the molybdenum balance figure was lower than for a dietary molybdenum intake of 132 ppm.

In experiments B(2), B(3) and B(4) the measurement of sulphate-reducing bacteria numbers was not consistent with the levels of rumen sulphide produced. A comparison of numbers of these bacteria was made (see Table 22) when the bacteria were grown in completely anaerobic conditions.

Strictly controlled conditions were set up by flushing the diluting medium of one per cent peptone solution and the growth medium

of Table 6 with nitrogen for 20 minutes before the rumen sample was added, whereas the others were not flushed. The bacteria were grown in control tubes containing the growth medium without molybdenum, tubes with medium plus 40 ppm molybdenum and tubes with medium plus 720 ppm molybdenum.

Table 22

The effects of flushing sulphate-reducing bacteria growth medium with nitrogen prior to incubation as compared with no flushing on the numbers of sulphate-reducing bacteria.

Level of molybdenum (ppm)	Treatment Sulphate-reducing bacteria numbers	
	With N-flushing	Without N-flushing
0	5.2×10^6	5.6×10^6
40	2.1×10^2	1.5×10^2
720	1.4×10^2	2.3×10^2

A similar pattern evolved as in the three experiments B(2), B(3) and B(4) in which sulphate-reducing bacteria numbers were measured. A significant difference ($P < 0.05$) occurred in both cases between the control levels (no molybdenum) and the two levels of added molybdenum, but there was no significant difference in bacteria numbers between the two molybdenum treatments or between the plus or minus molybdenum treatments. The effect of molybdenum was to significantly reduce the numbers of sulphate-reducing bacteria

irrespective of the treatment applied to the dilution medium and the growth medium, indicating that there was no ecological shift in the bacterial population from oxygen tolerant bacteria to oxygen sensitive bacteria.

Experiment B(5)

THE USE OF PURE CULTURE BACTERIA TO STUDY THE EFFECTS OF THE GROUP VI ANIONS ON SULPHATE REDUCTION *in vitro*.

Introduction

Although cell-free extracts of *D. desulphuricans* contain enzymatic systems that reduce thiosulphate, sulphite and tetrathionate to sulphide with molecular hydrogen (Ishimoto *et al.*, 1958), our initial attempts to obtain the reduction of sulphate in extracts were unsuccessful. The reduction of sulphate with hydrogen by whole cells is competitively inhibited by selenate; however, the reduction of sulphite or thiosulphate is not affected by the anion (Postgate, 1959). Moreover, molybdate ion similarly inhibits sulphate reduction but not the reduction of sulphite (Ishimoto *et al.*, 1954). Since Wilson and Bandurski (1958) observed that the enzyme ATP-sulphurylase catalyzes a rapid liberation of inorganic phosphate from ATP in the presence of inorganic pyrophosphatase and Group VI anions, the inhibition of sulphate reduction, but not sulphite reduction by MoO_4^{2-} and SeO_4^{2-} suggested that ATP-sulphurylase was involved in the reduction of sulphate by *D. desulphuricans*.

In experiments B(2) and B(4), lower levels of dietary molybdenum caused a significant increase in rumen sulphide production whereas higher dietary molybdenum intakes caused a depression in sulphide production. The reasons for this stimulation are unknown, and it was

0.1 ml of 10^{-4} ATP
1.0 ml of 0.04% <w/v>Na_2SO_4
0.1 ml of one of the solutions of group VI anions with concentrations ranging from 0,10,20,30,40 ,50,60,70,80,90,100,260.720 and 1440 ppm <w/v>

therefore decided to see if a similar situation occurred with *D. desulphuricans* and sulphate-reducing bacteria isolated from the rumen.

Materials and Methods

The pure culture *D. desulphuricans* was provided by Dr. P.A. Trudinger of the Baas-Becking Laboratory, Canberra.

The bacteria from the rumen of sheep fed a high sulphate diet were removed in a sample of rumen fluid per fistulam, the sample strained through gauze and then a series of dilutions from 10^{-1} to 10^{-8} were made, using bacteriological peptone as the diluting medium. One ml quantities of each of the dilutions were pipetted, in triplicate, into sterile 12 x 100 mm screw-capped culture tubes and incubated at 37°C in McIntosh and Fildes jars. The growth medium used was similar to that presented in Table 6.

A series of enrichments were carried out on the rumen bacteria until an almost pure strain was presumably achieved (Marshall, pers. comm.). This was then subjected to the same experimental procedure as the pure culture *D. desulphuricans*.

After four days in the growth media the bacteria were placed in a Warburg constant volume respirometer to determine sulphide production. 0.2 ml of bacterial culture were placed in the main compartment along with 10^{-4}M ATP, and varying concentrations of Group VI anions. 0.2 ml of 1N NaOH was placed in the centre well with folded filter paper, and 1 ml Na_2SO_4 in the side arm. The whole apparatus was flushed with nitrogen for 30 minutes before the sulphate

was tipped into the main well. Incubation was carried out for 1.5 hours at 39°C, after which the filter paper in the centre well was removed, placed in a test tube containing 2 ml of 1N NaOH, and 1 ml of Bismuth reagent (Dean, 1966) was then added. The resultant colour was measured in a Spectronic 20 at a wavelength of 400 nm.

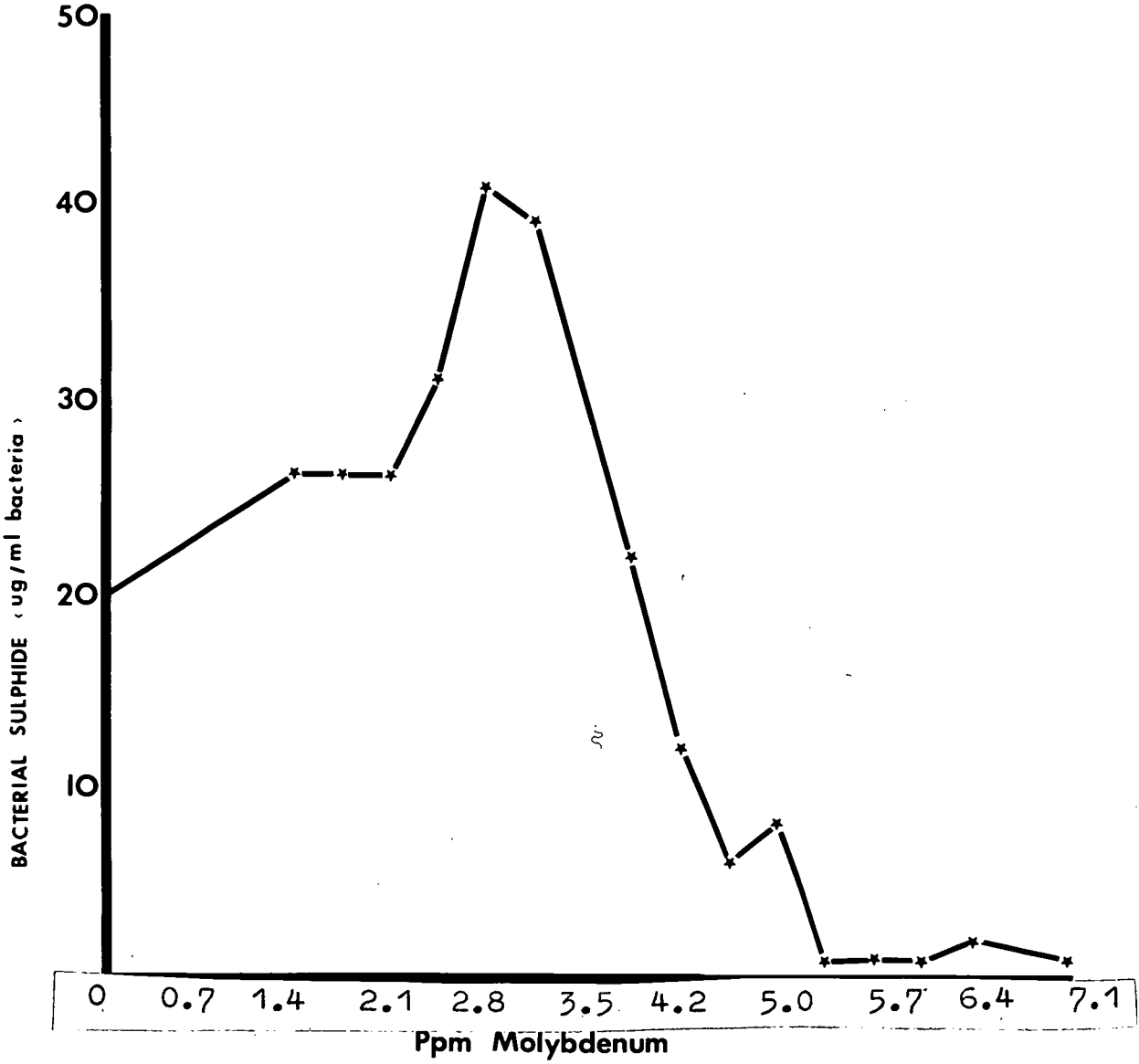
S^{35}-labelled sulphate was used as a check that the sulphide produced was from the introduced sulphate and not protein catabolism. After incubation the filter paper from the centre well was placed in the liquid scintillant of Herberg (1960), and counted in a Nuclear Chicago Model scintillation counter. Quenching was corrected by the use of internal standards.

Results

The levels of sulphide measured in the Warburg vessel were taken as evidence of the extent of sulphate reduction and the effects of varied dietary levels of molybdenum on bacterial sulphide production. The control level was that established without molybdenum. Blanks were also run, one without bacteria and one with added sulphate, and one with bacteria but without sulphate.

The reduction of sulphate by *D. desulphuricans* as evidenced by the production of sulphide is shown in Figure 25. Levels of molybdenum varying from 0.1 ppm to 4 ppm increased the sulphide production above that of the control, whereas levels of 4.5 ppm to 100 ppm depressed sulphide production. The three levels 18, 50 and 100 ppm are not

Fig. 25 The level of sulphide produced by *Desulphovibrio desulphuricans* after incubation for 1.5 hours in a Warburg Respirometer using varying concentrations of molybdenum in conjunction with Na_2SO_4 and ATP.


50
40
30
20
10
BACTERIAL SULPHIDE (ug/ml bacteria)
0
0.7
1.4
2.1
2.8
3.5
4.2
5.0
5.7
6.4
7.1
Ppm Molybdenum

shown in Figure 25 but these levels were 0.6, 0.4 and 0.4 µg sulphide per 0.1 ml bacteria respectively.

When S^{35}-labelled sulphate was used a similar pattern resulted (see Figure 26). The critical level of molybdenum in terms of depression of sulphide production lay between 3·5 and 4·5 ppm. Below this concentration the production of sulphide was greater than for the control level. In the rumen experiment. B(4) it was shown that 48 ppm molybdenum enhanced sulphide production and 76 ppm depressed it, so the critical molybdenum concentration appears to be of the same order (ALLOWING FOR RUMEN DILUTION FIG. 21) The relative activity of the S^{35}-labelled sulphate is presented in Table 23.

In the initial samplings the relative radioactivity remained constant (up to 4·5 ppm Mo), but with additions of 5 ppm Mo and greater the relative radioactivity increased markedly.

When group VI anions other than molybdate were used a different pattern of sulphide production occurred. At no stage during incubation of chromate, tungstate and selenate was a stimulatory effect above that of the control produced (see Figure 27). All concentrations of the anion tungstate depressed sulphide production below that of the control level, but from 2·1 ppm to 3·5 ppm a slight increase occurred. Selenate produced results similar to those of tungstate. Chromate depressed sulphide production, the higher the concentration of chromate the greater the depression. Thus it appeared from these results that of the group VI anions only molybdate stimulates sulphide production.

Fig. 26 The production of radioactive sulphide by *Desulphovibrio desulphuricans* after incubation for 1.5 fours in a Warburg Respirometer using varying concentrations of molybdenum in conjunction with 0.5 mCi of S^{35}-labelled sodium sulphate and ATP.

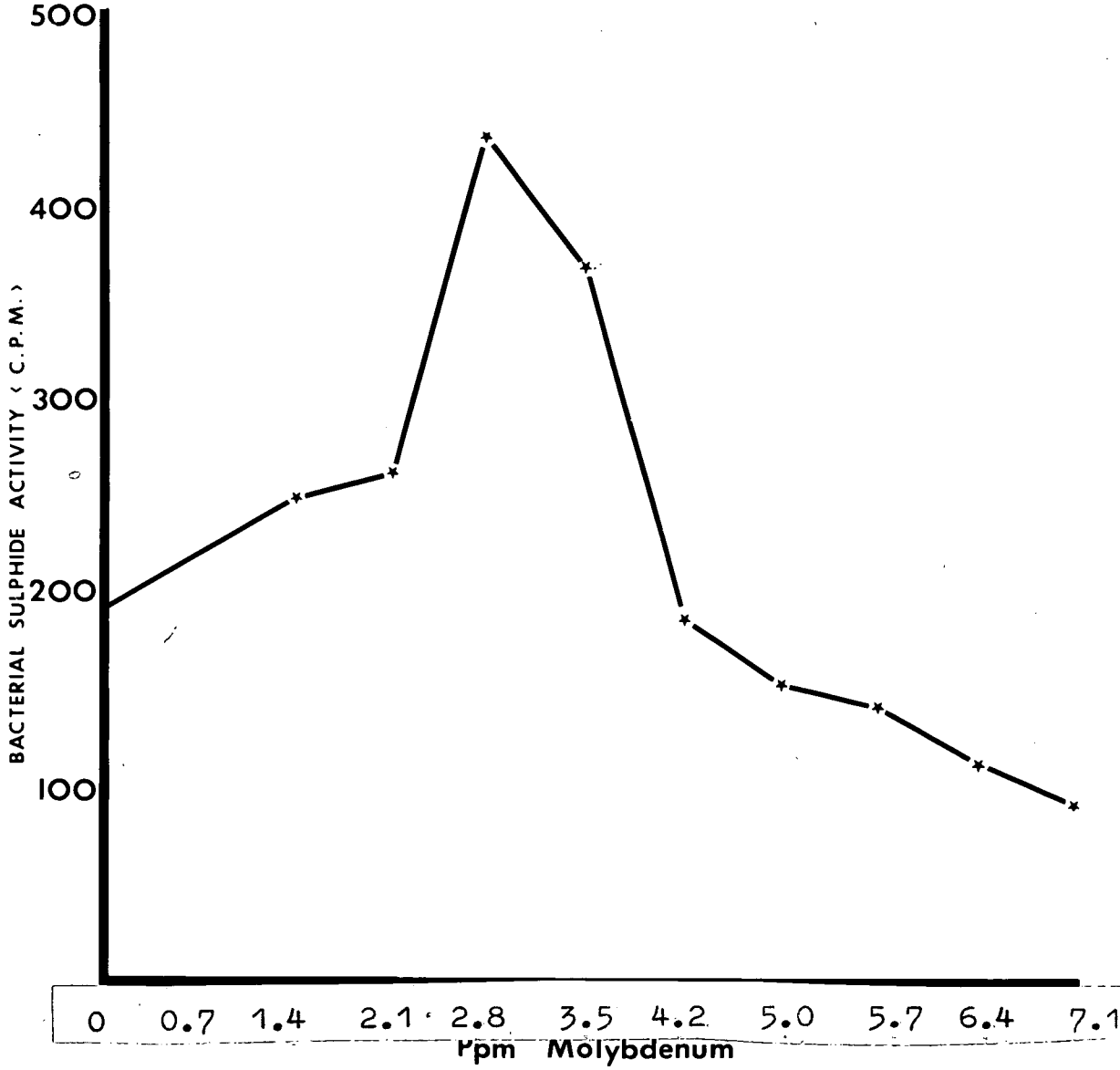
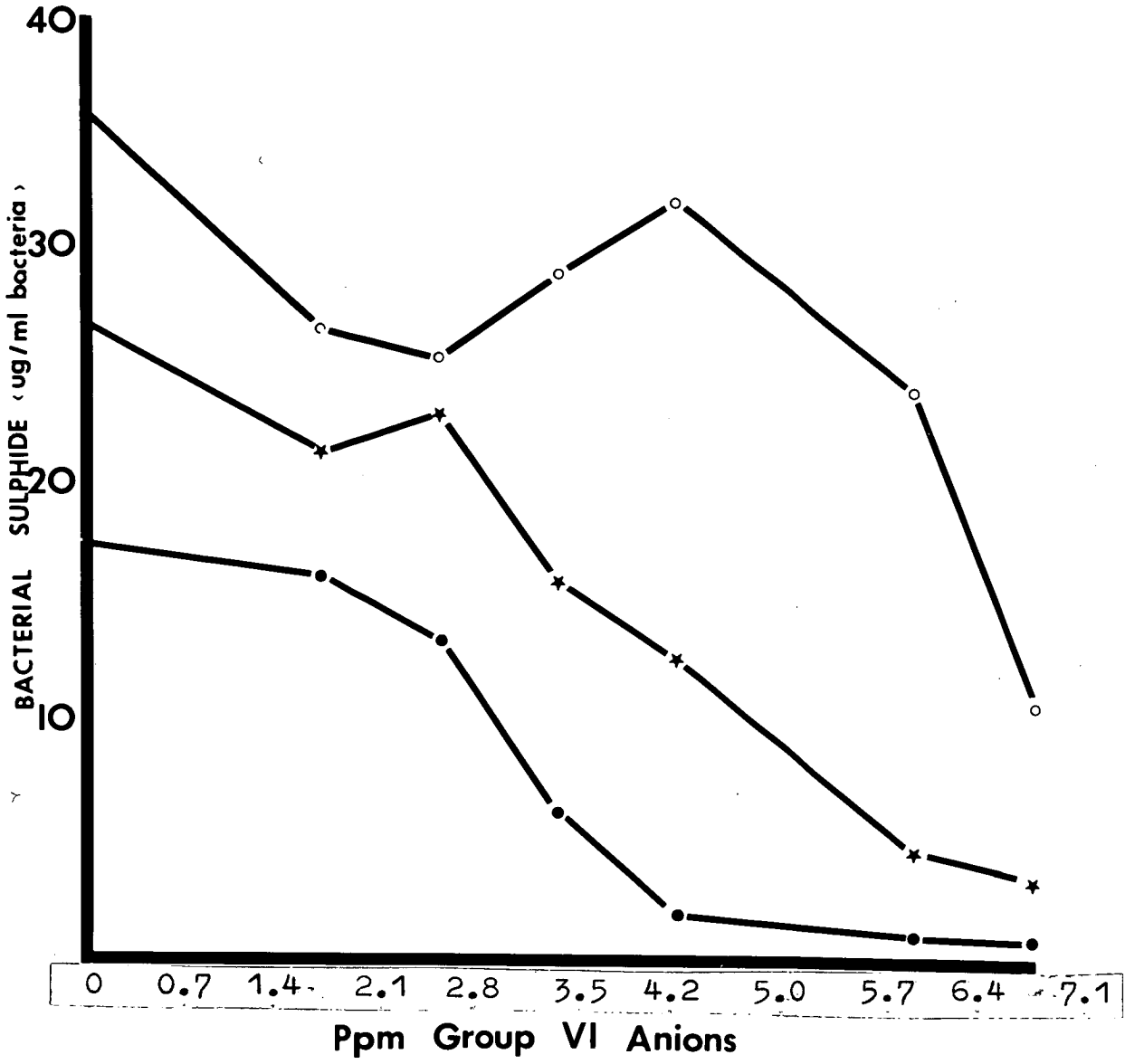

500
400
300
200
100
BACTERIAL SULPHIDE ACTIVITY (C.P.M.)
0
0.7
1.4
2.1
2.8
3.5
4.2
5.0
5.7
6.4
7.1
Ppm Molybdenum

Fig. 27 The level of sulphide produced by *Desulphovibrio desulphuricans* after incubation for 1.5 hours in a Warburg Respirometer using varying concentrations of the following group VI anions in conjunction with Na_2SO_4 and ATP: chromate ● —— ● ; tungstate ○ —— ○ ; selenate × —— × .


40
30
20
10
BACTERIAL SULPHIDE (ug/ml bacteria)
0
0.7
1.4
2.1
2.8
3.5
4.2
5.0
5.7
6.4
7.1
Ppm Group VI Anions

7/21/66

Table 23

Relative activities of sulphide produced by *Desulphovibrio desulphuricans* after incubation for 1.5 hours at 39°C with varying concentrations of molybdenum, and 0.5 mCi of S^{35}-labelled sodium sulphate.

Level of molybdenum (ppm)	Relative activity (CPS/µg sulphide)
0	9.6
0.7	9.6
1.4	10.0
2.1	10.6
2.8	12.3
3.5	16.0
4.2	19.4
5.0	145
5.7	58.0
6.4	92
7.1	

When the bacteria isolated from the rumen were used in the Warburg apparatus, a similar pattern of sulphide production was produced as that observed with *D. desulphuricans*. Figures 28, 29 and 30 show the sulphide production with sulphate, S^{35}-labelled sulphate and group VI anions other than molybdate.

The reduction of sulphide by anions other than molybdate showed a slightly different pattern from that with *D. desulphuricans*, in that a depression occurred.

Fig. 28 The level of sulphide produced by bacteria isolated from the rumen after incubation for 1.5 hours in a Warburg Respirometer using different concentrations of molybdenum in conjunction with sodium sulphate and ATP.

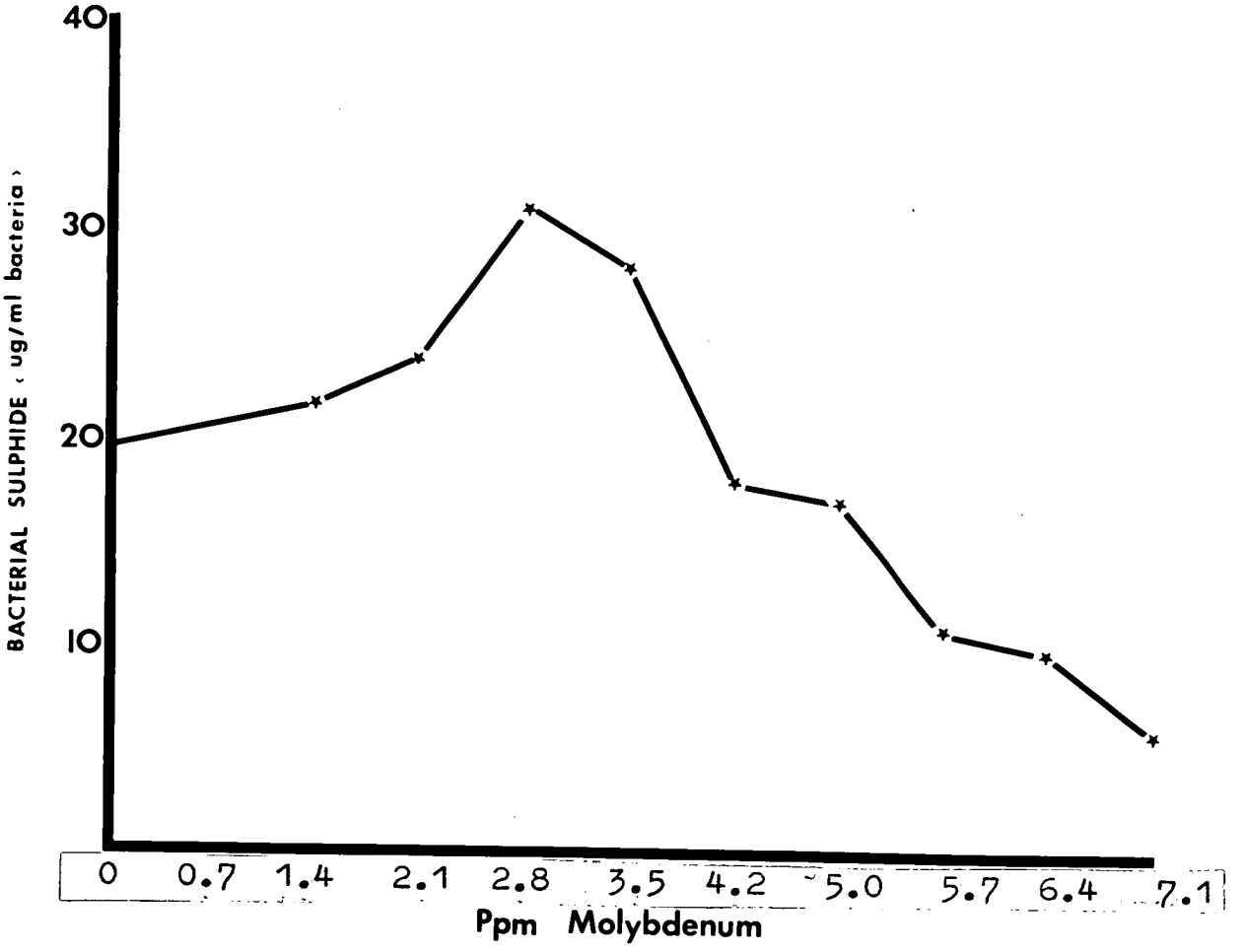

40
30
20
10
BACTERIAL SULPHIDE (ug/ml bacteria)
0
0.7
1.4
2.1
2.8
3.5
4.2
5.0
5.7
6.4
7.1
Ppm Molybdenum

Fig. 29 The production of radioactive sulphide by bacteria isolated from the rumen after incubation for 1.5 hours in a Warburg Respirometer using different concentrations of molybdenum in conjunction with sodium sulphate and ATP.

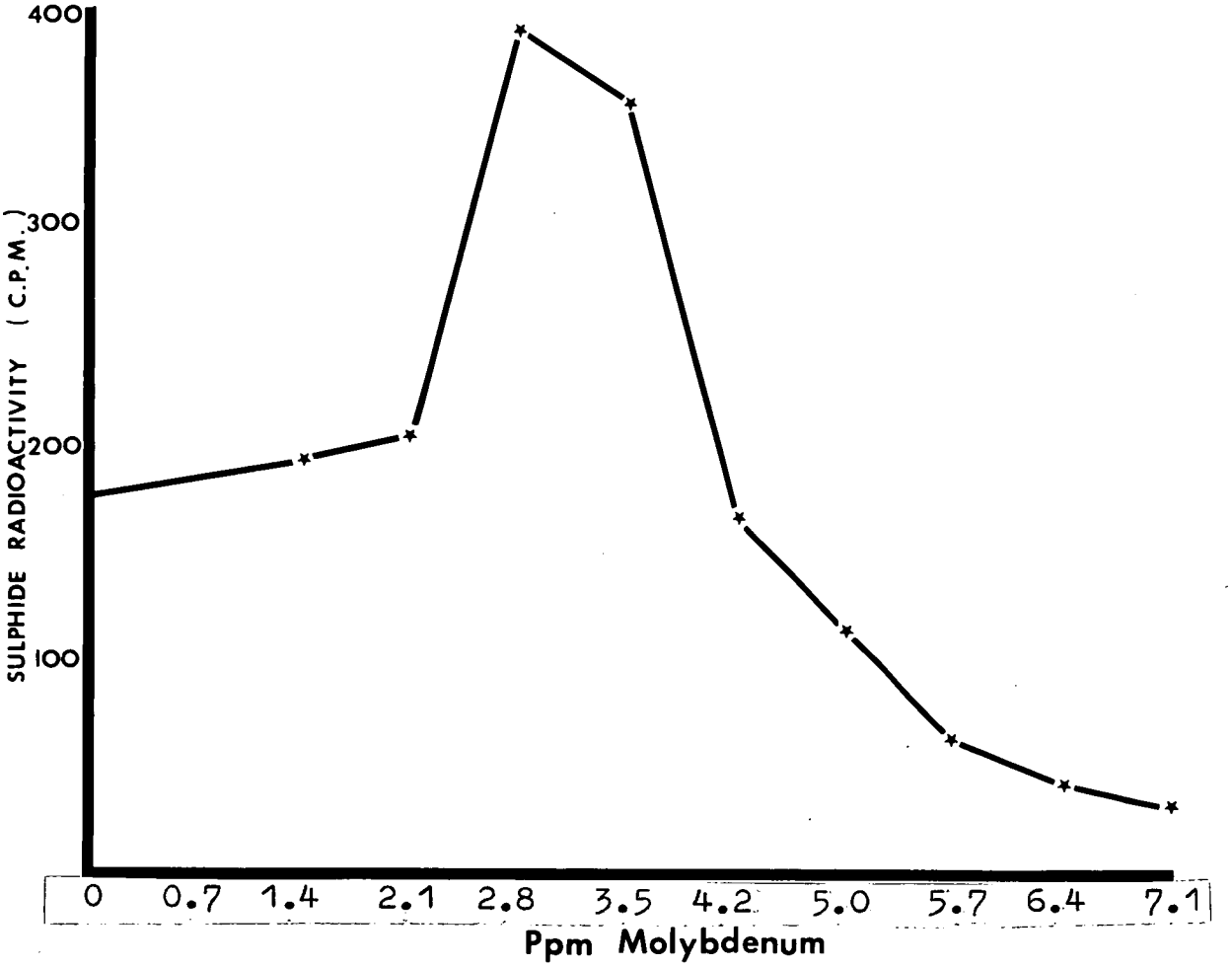
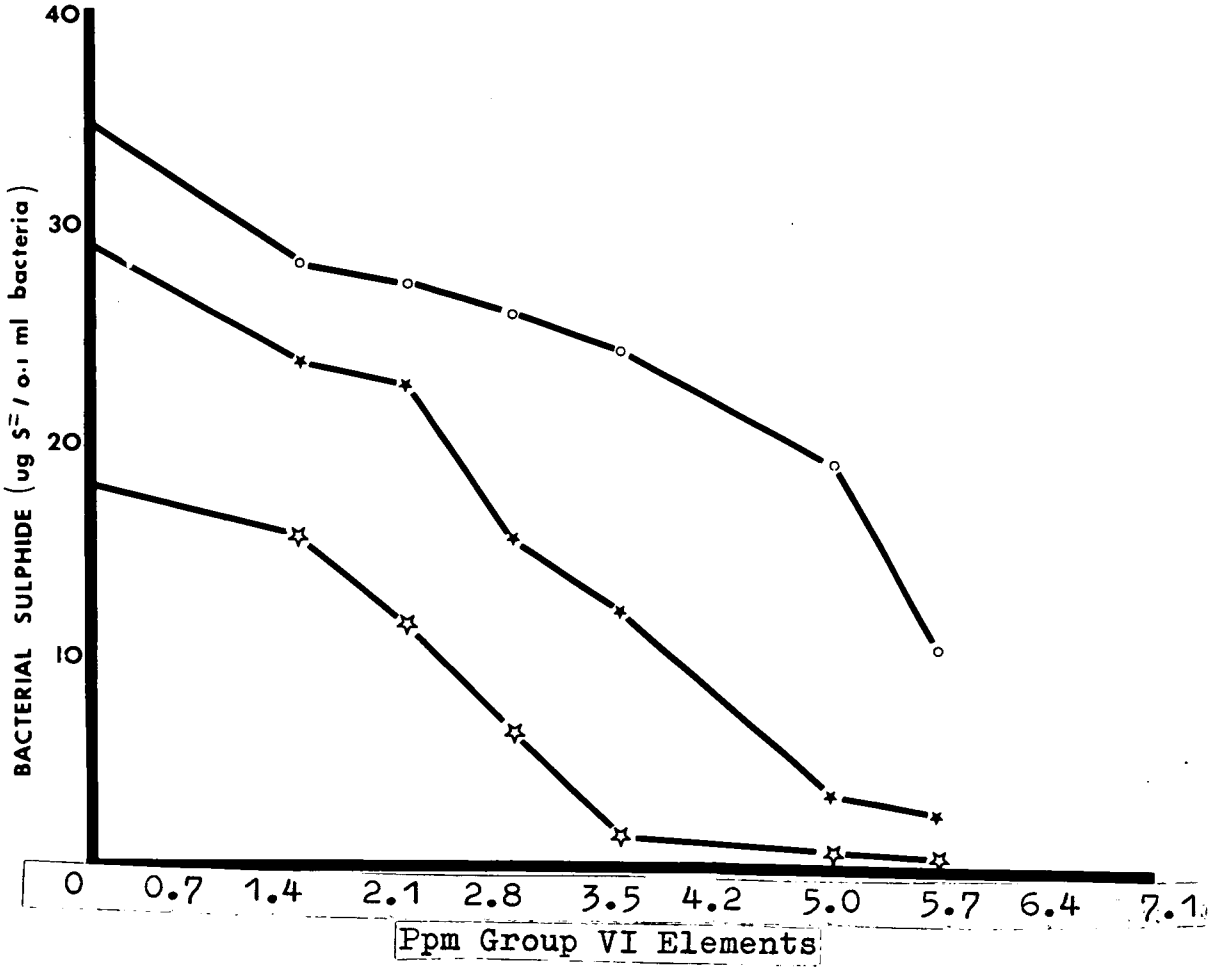

400
300
200
100
SULPHIDE RADIOACTIVITY (C.P.M.)
0
0.7
1.4
2.1
2.8
3.5
4.2
5.0
5.7
6.4
7.1
Ppm Molybdenum

Fig. 30 The production of sulphide by bacteria isolated from the rumen after incubation for 1.5 hours in a Warburg Respirometer using different concentrations of the following group VI anions in conjunction with sodium sulphate and ATP - chromate ✡—✡; selenate ×—×; and tungstate o—o.


40
30
20
10
BACTERIAL SULPHIDE (ug $S^{=}$ / 0.1 ml bacteria)
0
0.7
1.4
2.1
2.8
3.5
4.2
5.0
5.7
6.4
7.1
Ppm Group VI Elements

72/9/68

DISCUSSION

In section A data was presented demonstrating the presence of both APS and PAPS.

The presence of these two activated sulphate compounds in the rumen could be taken as evidence that inorganic sulphate was reduced via the microbial pathways proposed by Peck (1962). In such a pathway the first step is the activation of sulphate with ATP to form APS in the presence of the enzyme, ATP-sulphurylase. This investigation was originally planned to detect and isolate the enzymes involved in this pathway and then to measure their activity under different feeding systems. Attempts made to isolate the enzymes in our laboratory met with little success, probably because of the dilution one would expect in a mixed microbial system where only a small proportion of the population possesses sought after enzymes. The existence of mechanisms of sulphate reduction in the rumen other than enzymic reduction (e.g. chemical) could be postulated. The first step therefore in any investigation of rumen sulphate reduction is to determine if the microbial pathway of sulphate reduction is the only one involved. Wilson and Bandurski (1958), using enzymes isolated from plant tissue, reported that the group six anions - molybdate, selenate, tungstate, chromate and sulphite - all inhibited sulphate reduction by inhibition of ATP-sulphurylase. In view of these findings it was decided in our experiments to test the effect of the group six anions on sulphide production (or sulphate reduction) in the rumen.

In the preliminary experiment (section B1) it was shown that molybdate, selenate, tungstate and chromate depressed the levels of sulphide production in the rumen. However, only molybdate at the levels fed over the 14-day periods was without deleterious effects on the health of the sheep. Two levels of molybdate were used - 50 mg and 1.54 gm per day.

Mills (1960) reported that with sheep fed 10 gm of sulphate per day, a dietary intake of 50 mg molybdenum as molybdate per day increased rumen sulphide concentrations. At first such findings seem questionable as one would expect the opposite, since Wilson and Bandurski (1958) had shown the effect of group six anions to be one of complete inhibition. One could argue that because Mills (1960) fed his sheep twice daily and sampled at irregular intervals, the first sampling being two hours post-feeding, the initial peaks of sulphide production as obtained by Bray and Hemsley (1969) at 30 minutes post-feeding with sheep on a high intake of sodium sulphate were missed by Mills.

In the present investigations a level of molybdenum similar to that used by Mills, as well as higher levels were fed in the diet, and sampling was more frequent than in Mills' experiments. Complete inhibition of sulphate reduction (or sulphide production) was expected, but with 40 ppm molybdenum a stimulation of sulphide production occurred, thus supporting the findings of Mills (1960).

With all group six anions a level of 1.54 gm per day was sufficient to depress rumen sulphide production considerably below that of the control levels. Some anions were toxic, particularly chromate.

In view of these toxic problems it was decided in further work to use only molybdate, which had been shown the least toxic of these anions, and since the results obtained (Table 10) indicated that the effect of the other group VI anions on the depression of rumen sulphide was no greater than that of molybdenum. In subsequent experiments there were no signs of molybdenum toxicity, probably because it was not fed for long enough to cause poisoning. High intakes of sulphate in the diet (in this case 1.5 per cent sodium sulphate) reduce the retention of molybdenum (Dick, 1956) and so lead to the protection of the system against molybdenum toxicity.

The possibility existed that the group six anions were not directly responsible for the inhibition of sulphide production, but that the inhibition was in fact caused by the sodium cation of the salts used. Two sodium salts of chloride and bicarbonate were fed in similar sodium concentrations to those of the group six treatments to test this hypothesis. These salts had no effect on rumen sulphide concentrations as can be seen in Table 11. Over the experimental period of five hours very little variation in rumen sulphide concentration occurred between the basal treatment and the two treatments containing chloride and bicarbonate, whereas sodium molybdate at similar levels of sodium would strongly depress sulphide production over this period. Such findings support the proposition that the inhibition of sulphate reduction in the rumen is due to the group six anions and not sodium.

The dietary treatments used in later experiments (sections B2 and B4) had varying effects on the production of rumen sulphide. Levels of 40 ppm (section B2) and 20 and 48 ppm (section B4) all increased rumen sulphide production above that of the control. However, dietary intakes of 240, 720 and 1440 ppm molybdenum (section B2) and 76, 104, 132 and 160 ppm molybdenum (section B4) all had a depressing effect on rumen sulphide production. The critical level between stimulation and depression lay between 48 and 76 ppm molybdenum. Mills (1960) used only one level of dietary molybdenum (50 mg) whereas our experiments covered a range of molybdenum levels, thus enabling a dose response curve to be established.

The depression of rumen sulphide production below that of the basal level with the higher levels of dietary molybdenum (see Figures 5, 17 and 18) may have resulted from a number of causes, for example:

through 1. A molybdenum poisoning of general bacterial metabolism;

or 2. a fall in the reduction potential of the rumen contents and a consequent depression of chemical sulphate reduction;

or 3. an increased rate of sulphide absorption from the rumen;

or 4. the specific inhibition of the rumen sulphate-reducing bacteria by group six anions.

If the molybdenum caused a poisoning of the general bacterial metabolism, one would expect the following changes in the rumen system.

(a) a fall in total bacteria numbers;

(b) a depression of bacterial metabolism as indicated by a fall in total VFA production.

(c) a depression in the rate of cellulose digestion in the rumen.

The total bacterial count was not affected by molybdenum in experiments B(2), B(3) and B(4), where the total bacterial count was of the order of 10^{10} bacteria per ml. (Tables 15, 18 and 21) If the general bacterial system had been poisoned by the dietary molybdenum, then a fall in the total numbers would have been expected. Estimates of total rumen bacteria vary quite considerably with the counting techniques. Kohler (1940) studied numbers of total rumen bacteria by several techniques. By direct count he found approximately 1.3×10^{10} per gram, but by indirect means he calculated that there should be greater than 10^{11} per gram. However, Kohler (1940) was able by aerobic culturing to grow only 2.5×10^{7} organisms per gram. Gall *et al.* (1949), using a direct slide count found bacteria in numbers ranging from 5-10 $\times 10^{10}$ per gram of fresh rumen contents. Munch-Peterson (1964) suggested that more comprehensive chemical analyses of rumen material are needed, especially for the formulation of better culture media for rumen bacteria. In our experiments a direct counting technique was used throughout and relatively constant numbers were counted. It was not possible to determine if there was any change in the relative abundance of any microbial species, but there was no significant treatment effect on total numbers.

Since the total bacterial numbers remained relatively constant throughout, a further indication of the poisoning of the general bacterial metabolism by molybdenum may have been indicated by the interruption of rumen bacterial metabolism which would show as a change in the total VFA production. Steam volatile fatty acids were measured at each sampling time during all experimental periods. At no stage was there any significant change in the levels of VFA caused by changes in dietary molybdenum intake (Table 12 and Figures 4, 12 and 21).

It is well established that acetic, propionic and butyric acids produced by fermentation of food in the rumen are major sources of energy for ruminants; but precise measurement of volatile fatty acid production remained in a relatively unsatisfactory state (Rook, 1964; Warner, 1964) until Leng *et al.* (1968) used radio-isotope techniques to derive regression equations relating entry rates to rumen concentrations. In practice, the integration of estimated entry rates made for short periods of time would be achieved most simply by analysing a sample of digesta from continuous automatic collection or comprised a number of subsamples taken at regular intervals. Measurements of this type have been made by Gray *et al.* (1966, 1967) and Weller *et al.* (1967) but most of their results were not obtained under steady-state conditions. Studies of absorption of VFA from the rumen, conducted by adding solutions of VFA to the washed out rumen have shown that the rate of absorption is directly related to the VFA concentration (Masson and Phillipson, 1951). Rumen concentration of VFA is proportional to the production of these VFA in the rumen in our experiments.

The concentrations of VFA did not differ significantly between treatments and since the production of VFA in the rumen is proportional to concentration it would appear that there was no effect of molybdenum at any concentration on total VFA production.

Although relative proportions of acetic, propionic and butyric acids in the total volatile fatty acids were not examined a change in the proportions of these acids may have occurred without affecting the total volatile fatty acid concentration. In experiments B(2) and B(4) the level of sodium sulphate in the diet was constant, whereas in experiment B(3) the level of sulphate was varied from zero to 1.5 per cent of the total ration. Whanger and Matrone (1965) indicated that levels of propionate, butyrate and higher fatty acids were higher in the rumen fluid from sheep fed a sulphur deficient purified diet with sodium sulphate supplements than from the rumen fluid of sheep fed the sulphur deficient diet alone. These authors found only traces of lactic acid in the rumen fluid of sulphur-fed animals, in contrast to a large amount of lactic acid in that of sulphur-deficient animals, and suggested, that the micro-organisms from the sulphur-fed animal can synthesize butyrate and higher fatty acids from acetate whereas the micro-organisms from the sulphur-deficient animal cannot.

Cellulolytic activity was measured by the cotton thread technique. It was found that the rate of cellulose digestion was not significantly affected by the dietary molybdenum treatments in diets which contained a similar amount of sulphate (sections B2 and B4), whereas a change in the level of dietary sulphate either with or

without molybdenum did cause a significant change in the rate of cotton thread disappearance (section B3). The depressing effect of readily available carbohydrates on fibre digestion when the ration is deficient in nitrogen is well known (Hamilton, 1942; Burroughs *et al.*, 1949 El-Shazly *et al.*, 1961). In our studies the level of nitrogen intake was constant in all experimental work. There is evidence (Belasco, 1956; Cline *et al.*, 1958) that the increased rates of cellulose digestion *in vitro*, in the presence of adequate nitrogen and small amounts of carbohydrates other than cellulose, are associated with increased levels of higher VFA in the fermentation mixture. In the present investigations (section B3) a deficiency of sulphate in the diet irrespective of the presence or absence of molybdenum caused a highly significant ($P < 0.001$) depression in the rate of cotton thread digestion (see Table 18). This was associated with a decrease in the level of total VFA in the rumen from 67.0 m moles per litre of rumen fluid in sheep fed a sulphate supplement to 60.5 m moles per litre in sheep fed a diet minus sulphate. However, in experiments B(2) and B(4) (Tables 15 and 21) the addition of dietary molybdenum had no significant effect on the cotton thread digestion. A number of workers (Williams and Moir, 1951; Bray and Hemsley, 1969; Coombe *et al.*, 1971) have demonstrated that the primary effect of sulphur deficiency appeared to be the limitation of rumen microbial growth and metabolism.

It would therefore appear that molybdenum did not materially affect general bacterial metabolism since there was no effect of molybdenum at any concentration on the size of the rumen bacterial

population, or on fermentation activity as would be shown by a change in VFA production or in cellulolytic activity.

The depression of rumen sulphide production below that of the basal level with the higher levels of dietary molybdenum may have been due to a fall in the reduction potential of the rumen contents and a consequent depression of chemical sulphate reduction as distinct from enzymic. However, the addition of molybdenum in the diet had no effect on the redox potential of the rumen. The range of redox potentials ranged from -290 mV to -380 mV throughout the experimental period when measured in the rumen. The question thus remains - will a reducing system of this magnitude reduce sulphate to sulphide without specific enzyme catalysis? The reduction potential values measured in the rumen are low enough to maintain a very anaerobic system and it becomes very difficult to create an *in vitro* environment identical with that of the normal rumen (Broberg, 1957). Values of redox potentials of sulphate are not well documented, and consequently it is not an easy matter to compare the values measured *in vivo* to those measured in an efficient *in vitro* system. The only information concerning redox potentials under conditions which in some respects resemble those in the rumen is that of Marston (1948) giving the potential during cellulose fermentation *in vitro* as -380 mV at pH 7.0 with variations up to ± 30 mV. In our studies the potential was of the order of -330 mV at pH 6.8 with variations up to ± 40 mV.

The possibility exists that the rumen bacteria by producing a reduction potential of -350 mV, enables chemical reduction to take place. Throughout all the experiments there was no significant difference in the reduction potential between treatments. At all stages the redox potentials increased with time between T0 to T210 and then returned to pre-feeding levels (Figures 9, 15 and 23). This increase may have been due to the inevitable addition of oxygen through the fistula and with the feed.

Broberg (1957) stated that measurements of redox potential in rumen contents could give a basis for the estimation of the microbiological activity. In our studies it could be argued that there was no change in microbiological activity since there was no difference in reduction potential between treatments.

These results indicated that there was no fall in reduction potential of the rumen contents and thus there should have been no effect on chemical sulphate reduction if it does occur in the rumen.

An increased rate of sulphide absorption from the rumen could lead to a depression in rumen sulphide production. Two possible mechanisms may be involved, viz. an increase in sulphide concentration in the rumen leading to an increase in absorption rate, and secondly andecreasein pH values leading to an increased rate of sulphide absorption across the rumen wall (Bray, unpublished data).

Rumen daily pH patterns were not affected by molybdenum. Such findings are supported by VFA production being unaffected by molybdenum treatments since rumen pH and rumen VFA production are strongly correlated.

Although Bray (unpublished data) has shown that more acid pH favours a faster rate of sulphide absorption from the rumen, such an effect could not be responsible for lower rumen sulphide levels in the high molybdenum treatments since rumen pH was unaffected. These values lay in the range of 6.7 to 7.5. Under normal conditions the pH of rumen contents varies with time (Monroe and Perkins, 1939; Briggs *et al.*, 1957; Brethour *et al.*, 1958). The greater proportion of this variation is due to volatile fatty acid formation (Monroe and Perkins, 1939; Phillipson, 1942; Chance *et al.*, 1953).

The first three propositions already discussed have been eliminated as causes of depression of rumen sulphide below that of the basal diet. The total rumen bacterial numbers remained relatively constant throughout the experiments, there was no depression in the reduction potential of the rumen contents and no consequent depression of chemical sulphate reduction, and there was no significant increase in rumen pH due to treatment. The fourth proposition must now be considered as being the most likely cause of the depression of rumen sulphide production, this being the specific inhibition of rumen sulphate-reducing bacteria by group six anions.

In all experiments the sulphide levels were used as an indication of the level of sulphate reduction. As molybdenum was added in increasing concentrations the effect on rumen sulphide production varied, but the effect on general rumen metabolism remained the same. In experiment B(2) 40 ppm molybdenum increased the level of sulphide above that of the control level whereas the higher dietary levels of

molybdenum (240, 720 and 1440 ppm) severely depressed rumen sulphide production. Again, in experiment B(4) dietary levels of 20 ppm and 48 ppm molybdenum increased rumen sulphide production whereas dietary intakes of 76, 104, 132 and 160 ppm molybdenum depressed rumen sulphide below that of the basal ration. The critical level of added molybdenum was between 48 ppm and 76 ppm in terms of inhibition of sulphate reduction (see Figure 18). The higher dietary levels of molybdenum, while significantly depressing rumen sulphide below that of the control diet, did not completely stop the production of sulphide. This small level of sulphide production may have been due to autolysis of bacterial, dietary and salivary protein.

In section B(3) we tested the possibility of molybdenum increasing sulphide release from bacterial protein by comparing the effects of diets containing plus and minus sulphate and plus and minus molybdenum. In the absence of sulphate the production of sulphide in the rumen was virtually zero and the result was unaffected by the presence or absence of molybdenum.

The addition of molybdenum in our experiments to diets deficient in sulphur did not have any effect on the bacterial protein since the sulphide production with and without molybdenum was virtually zero. When sulphate was added to the diet a substantial increase in sulphide production was evident. The effect of molybdenum in diets containing sulphate was to depress rumen sulphide production, but not to the level of those diets minus sulphate.

The numbers of sulphate-reducing bacteria were measured during the experimental work in sections B(2), B(3) and B(4), and the numbers when sheep were fed the basal diet were estimated to be an average of $6.5 \times 10^6 \pm 3 \times 10^5$ per ml when counted by the most probable number techniques. Determination of the viable count is facilitated by the formation of black colonies through the formation of ferrous sulphide. Surface counts are not used usually since the bacteria grow poorly on an agar surface. The period required for turbidity or colonies to develop at 30° has been reported to vary between 3 and 28 days (Grossman and Postgate, 1953; Alico and Liegey, 1966). In our studies a constant time period of five days was used for all sulphate-reducing bacteria. When molybdenum was added to the diet, regardless of the level, the numbers of sulphate-reducing bacteria fell to an average value of $6 \times 10^2 \pm 20$ per ml, over the course of three experiments (B2, B3 and B4), viz. counts of the order of 10^6 per ml when sheep were fed the basal diets and counts of the order of 10^2 per ml when molybdenum was added in the diet. Obviously the molybdenum was having an apparent depressing effect on the numbers measured although low levels of dietary molybdenum increased rumen sulphide production. These numbers measured when dietary molybdenum was added, when considered in terms of the whole rumen volume, would not have been sufficient to produce such large quantities of rumen sulphide. An estimate of the minimum number of bacteria required to produce such levels of sulphide was made from the *in vitro* studies, this estimate being 4×10^4 bacteria per ml. Obviously some other factor must have been responsible for this

increase - one which was not detected with the most probable number counting technique.

It is difficult to obtain uniform bacteria samples from the rumen (Warner, 1962). Rumen contents are a heterogeneous mixture of solids and liquid, often with marked stratification of the solids. The "free" micro-organisms are suspended in, and are usually considered with, the liquid portion. In straining through gauze, muslin etc., the feed particles mat together rapidly and form an efficient filter for at least the larger organisms if special precautions are not taken. Boyne *et al.* (1956) used a method of wet sieving that largely obviated this difficulty, but resulted in inconveniently large volumes of fluid in the sample which then had to be concentrated.

However, this does not explain the reduction of numbers of sulphate-reducing bacteria when molybdenum was added to the diet. A number of possible explanations can be advanced to explain this phenomena, viz.

1. Changes in the rumen bacterial population allowing assimilatory sulphate reducers to reduce sulphate in preference to some dissimilatory reducers.
2. Increased reaction rate induced by an increased removal of inorganic pyrophosphate caused by molybdenum.
3. The existence of sulphate-reducing bacteria in the rumen having different nutrient requirements.

The dissimilatory reducers, *D. desulphuricans* and *Desulphotomaculum nigrificans* would be expected to be present in the rumen, since these are the classic dissimilatory sulphate reducers. However, the addition of molybdenum to the rumen could cause an ecological change in rumen microbial population, allowing assimilatory bacteria to reduce sulphate in preference to some of the dissimilatory reducers. The synthesis of the enzymes reducing inorganic sulphate to sulphide in *E. coli* (an assimilatory reducer) is under repressive control by the end product of sulphate reduction, L-cysteine (Pasternak, 1961, 1962; Ellis *et al.*, 1964). It is commonly found in bio-synthetic pathways that the end product inhibits the activity of the first enzyme in the sequence (Umbarger, 1961). However, L-cysteine and some related sulphur compounds were found not to inhibit the enzymes catalyzing the synthesis of PAPS from sulphate and ATP (Ellis *et al.*, 1964). Similarly the enzymes reducing PAPS to inorganic sulphite, and sulphite to sulphide are not inhibited by L-cysteine. The assimilatory reducers do not normally produce any appreciative quantities of sulphide, and unless the sulphate-reducing bacteria are able to adapt themselves to grow with molybdenum as substrate, then there is no reason to believe that any change takes place.

If an ecological shift does not occur, a further possibility exists in that low levels of molybdate may have increased the removal of inorganic pyrophosphate leading to an increased stimulation of the activity of the enzyme pyrophosphatase. Such an increase would increase the forward rate of reaction and thus increase the rate of

activation of sulphate. However, it was not possible under the conditions of the experiment to measure the removal rate of inorganic pyrophosphate, but the normal rumen function would be expected to produce the pyrophosphatase which removes the pyrophosphate.

A more likely explanation is the existence of sulphate-reducing bacteria in the rumen having different nutrient requirements. The possibility of two types of sulphate-reducing bacteria being present in the rumen - those which are sensitive to oxygen and those which are tolerant to oxygen - could exist, and the addition of molybdenum causes a shift to the oxygen sensitive bacteria. All the bacteria were grown anaerobically, but during the dilution process duplicates were run, one lot being flushed with nitrogen while the other lot were not. It was thought that the most probable number technique may not have been efficient enough to count the sulphate-reducing bacteria when molybdenum was added under the ordinary anaerobic conditions. If a shift to oxygen sensitive bacteria occurred then flushing the medium with nitrogen may have been sufficient to detect such a change. Bacteria were grown both with and without molybdenum, and both the dilution medium and growth medium were flushed with nitrogen in some cases while not in others, but no differences in numbers counted were obtained. Two levels of molybdenum were used plus a control, and in both cases where molybdenum was added the numbers of sulphate-reducing bacteria dropped from 10^6 per ml to 10^2 per ml. Preliminary experiments of Mara and Williams (1970) indicated that the inclusion of 0.1 per cent (w/v) of both of the

reducing agents ascorbic acid and sodium thioglycollate made little difference to the viable count with some media, but with other media viable counts were improved by this addition. In our studies initial trials both with and without reducing agents showed no difference in counts, and because of the statement of Mara and Williams (1970) that sodium thioglycollate can be toxic at certain concentrations it was decided not to continue with this agent to reduce the possibility of toxicity.

A further suggestion as to the failure to count the sulphate-reducing bacteria when molybdenum was added could be that the sulphate-reducing bacteria have a specific nutrient requirement which is present in the rumen but which is removed during the dilution process. These bacteria may be stimulated by the addition of molybdenum in the diet, and although they grow normally in the first two dilutions (i.e. to 10^{-2}), the greater nutritional requirement may be removed at greater dilutions to such an extent that the bacteria will not grow. This would suggest that there is an ecological change in the sulphate-reducing bacterial population. Further experimental work is necessary to substantiate this statement, but at present this appears the most likely of the propositions (Marshall, pers. comm.).

If the reduction in total dissimilatory sulphate reducers is not an artifact then the question arises - why aren't all the bacteria destroyed by dietary molybdenum? The work of McNaught *et al.* (1950) and Martinez and Church (1970) suggests that *in vitro* rumen bacteria tolerate quite high concentrations of molybdenum (up to 500 ppm)

without serious impairment of function. In the present studies the maximum concentration of molybdenum achieved in the rumen (in the vicinity of 100 ppm) of sheep fed a diet containing 1440 ppm molybdenum was within the tolerable levels suggested by McNaught *et al.* (1950) and Martinez and Church (1970). These workers did, however, report an increase in cellulose digesting bacteria activity with levels of 300 ppm molybdenum, and so it was possible that a similar stimulation of the sulphate-reducing bacteria could have occurred with rumen levels in the vicinity of 2 ppm with an intake of 20 ppm, 3 ppm with an intake of 40 ppm and 3.5 ppm with an intake of 48 ppm molybdenum.

The stimulation of rumen sulphide production by low levels of molybdenum in the diet as described by Mills (1960) has been substantiated by our results. However, the reason for this stimulation has not been explained by Mills or by us. The numbers of sulphate-reducing bacteria in the rumen measured in the present investigations could not be considered as being the contributing factor. The thought that this increase was an artifact within the rumen was considered and so an *in vitro* technique using a Warburg respirometer was designed to check the activity of both pure cultures of *D. desulphuricans* and sulphate-reducing bacteria isolated from the sheep rumen. These experiments showed that both forms of bacteria increased sulphide production in response to low molybdenum levels in the media above that of zero molybdenum or high levels of molybdenum. These results indicated that the sulphate-reducing bacteria were apparently responsible for the effect in the rumen. The sulphate-reducing bacteria form a specialized

group of microbes that use sulphate as a terminal electron acceptor for their respiration. Though many microbes generate sulphide metabolically, sulphate often being the primary source of that sulphide, the process is normally a small scale one involving the incorporation of sulphur into cell protein and its subsequent degradation by catabolic and autolytic processes. Although the work of Wilson and Bandurski (1958) and Hilz *et al.* (1960) showed that the group six anions completely inhibited sulphate reduction by inhibiting the activity of the enzyme ATP-sulphurylase, these workers used concentrations of 4×10^{-4} M of group six anions. Such concentrations correspond to the higher levels of molybdenum used by us; Wilson and Bandurski (1958) and Hilz *et al.* (1960) did not state whether they had studied the effects of lower concentrations. Mills (1960) presented results indicating that lower levels of molybdenum did stimulate rumen sulphide production but did not offer any explanation as to the cause, nor did he use any other levels of molybdenum. Our results have shown that the stimulation of sulphide production with low levels of molybdenum, varying from 20 ppm to 48 ppm in the DIET and 0.7 ppm to 4 ppm *in vitro* increased the sulphide production above that of the control, whereas levels of 4.5 ppm to 100 ppm in both cases depressed sulphide production with both the *D. desulphuricans* and the enriched rumen cultures. The critical level of molybdenum in both the rumen and *in vitro* lay between 3.5 ppm and 4.5 ppm per ml. These *in vitro* results substantiate the theory that the rumen bacteria were responsible for this unusual behaviour.

In the rumen studies, the levels of total sulphate sulphur in the rumen showed the reverse pattern to sulphide levels in all sections of the experimental work. The levels of sulphate all declined with time over the 8.5 hour sampling period from an average maximum of 140 µg at T30 to an average minimum of approximately 60 µg per ml at T510. This fall in concentration of rumen sulphate with time would be expected. However, the rate of reduction was not as rapid as in experimental section A. As the level of molybdenum in the diet increased, the rate of removal of sulphate from the rumen decreased. This would be expected since the higher levels of molybdenum inhibited the production of rumen sulphide. Bray (1969b) found that sulphide is the only form of inorganic sulphur likely to be absorbed in any quantity from the rumen and as a result the higher levels of sulphate in the rumen would be due to a slower rate of disappearance.

In experiment B(3) during the treatment of minus sulphate plus molybdenum there was a tendency towards a slightly lower feed intake, although the difference was not significant. When sulphate was added to the diet the intake increased marginally. The faeces weights for minus sulphate plus molybdenum were less than for the other treatments. Similarly the urine volumes were higher in this treatment due presumably to a molybdenum induced diuresis. Associated with this molybdenum induced diuresis was an increased excretion of nitrogen in the urine. Neither plasma nor urinary urea and ammonia levels were measured in this study. Thornton (1970) suggested that the urinary excretion of urea may not be governed by, but rather contributes to the elevated urine flow.

However, Thornton's work was carried out with diets which were not loaded with molybdenum and so it is difficult to compare his results with ours. The effects of molybdenum treatment in urinary nitrogen output were significant leading to a highly significant difference ($P < 0.001$) due to treatment.

In experiment B(3) the total sulphur output decreased when molybdenum was added to the diet containing added sulphate. This increase in sulphur retention was reflected in a decrease in both the urinary and faecal sulphur outputs. This was contrary to the findings of Scaife (1956) who found an increase in sulphate excretion in the urine of sheep on high molybdenum intakes (10-50 mg Mo per day), and Scaife suggested that sulphate was an end-product of increased protein catabolism. McCarter *et al.* (1962) reported that molybdate promotes protein catabolism and as a result it would be expected that the concentration of sulphate would increase with molybdate feeding.

The levels of molybdenum measured in the rumen were positively correlated with the levels fed in the diet. The data presented in Figures 6, 13 and 21 indicate the level of molybdenum measured in the rumen. These curves are flatter than expected as it was thought these levels would decrease with time over the 8.5 hours sampling period. However, a period of ten days was necessary to completely eliminate all traces of molybdenum from the sheep's digestive system. The reason for such a slow rate of removal is not clear, particularly when the release of Cr_2O_3 used in digestibility trials has been shown by Lambourne (1957), Lambourne and Reardon (1963) and Troelsen (1965) to

be cleared from the system by a maximum of three days. Possibly the molybdenum is recycled in the saliva, and as a result the molybdenum remains within the system for this longer period of ten days.

The change in dietary intake of molybdenum was reflected by a significant difference in molybdenum balance figures in both experiments B(3) and B(4). These values were expected in experiment B(3) in which diets A and C had no molybdenum, but in experiment B(4) there was a steady increase in molybdenum balance in all diets except the one with 160 ppm, when a substantial decrease in the molybdenum balance was obtained. This was due to an increase in the level of faecal molybdenum relative to that of urine. This contrasts with the work of Comar *et al.* (1949) who fed labelled sodium molybdate to a steer and this resulted in 34 per cent of the dose appearing in the faeces and 45 per cent in the urine within 14 days when given by mouth and 11 per cent in the faeces and 37 per cent in the urine within six days when injected intravenously. In experiment B(3) the balance figure in diet B (minus sulphate plus molybdenum) was substantially higher than in diet D (plus sulphate plus molybdenum). This was due to an increased output of molybdenum in both urine and faeces with diet D. The addition of sulphate to the diet had the effect of increasing the molybdenum output. Such an effect of sulphate may have been the reason that no molybdenum toxicity occurred. Prolonged intakes of diets high in molybdenum have been shown to have serious effects on the health of animals and the interaction of molybdenum, copper and sulphate in animal nutrition is well known (reviewed by Underwood, 1971).

One aspect of molybdenum poisoning that needs emphasis is that invariably a depression of food intake occurs. This may in turn cause elevation or depression of body enzyme levels, excretion rates and sensitivity of the body to various metabolites. In experiment B(3) a depression in food intake occurred in sheep on the molybdenum intake without sulphate, and it was in this group that urine volume increased.

General Summary and Conclusions

The reduction of inorganic sulphate in the rumen of sheep has been studied. The use of S^{35}-labelled sodium sulphate was made to isolate the intermediates APS and PAPS from the sheep's rumen after feeding a diet in which added sodium sulphate was the only source of sulphate. Having established the presence of these two intermediates, more detailed examinations of the pathway of sulphate reduction were carried out.

Group VI anions were incorporated in the diet and their effects on both sulphate reduction and general rumen metabolism were studied. Following preliminary studies with molybdate, tungstate, chromate and selenate, it was decided to use only molybdate for reasons discussed in experimental section B. A summary of the findings are considered below.

1. Levels of 20 ppm, 40 ppm and 48 ppm molybdenum fed as molybdate all stimulated rumen sulphide production above that of a basal level, while levels ranging from 76 ppm to 1440 ppm molybdenum inhibited sulphide production below the basal level.

2. General rumen metabolism was not disturbed when molybdenum as molybdate was fed. This was shown to be true by measurement of rumen pH, rumen redox potential, steam volatile fatty acid production and total bacterial counts. If the general metabolism had been disrupted, a change in the pattern of one or all of these parameters would have been expected.

3. Sulphate-reducing bacteria numbers were measured, but the numbers counted did not correspond with the stimulation of rumen sulphide production on the low levels of molybdate feeding. The reasons for this are discussed.

4. Rumen sulphate levels were measured, and found to be correlated with rumen sulphide levels. As the level of molybdenum in the diet was increased the production of rumen sulphide decreased, and associated with this was a corresponding decrease in the rate of disappearance of sulphate from the rumen.

5. The reduction of sulphate by both *Desulphovibrio desulphuricans* and sulphate-reducing bacteria isolated and enriched from the rumen was studied. A similar pattern of response was obtained as in the rumen studies and as a result the conclusion was reached that the reduction of sulphate in the rumen was carried out by the sulphate-reducing bacteria and not by chemical reduction.

From these observations both in the rumen and *in vitro* it can be concluded that the reduction of inorganic sulphate in the rumen of sheep follows the microbial pathway involving the initial activation of sulphate by ATP, and that the enzyme ATP-sulphurylase is responsible for this activation since the group VI anions can inhibit the activity of this enzyme without disturbing the general rumen function.

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Appendix 1. Experiment A.

Analysis of variance of rumen sulphate levels.

Due to	df	S.S.	M.S.	F
Sheep	2	23222.8	11611.4	4.24 *
Time	4	66414.9	16603.7	6.07 *
Error	8	21861.9	2732.7	
Total	14	111499.6		

* = $P < 0.05$.

Appendix 1. Experiment A.

Radio activity (counts per minute) of selected regions of chromatography strips used in the isolation of APS and PAPS from rumen fluid sampled at five minute intervals from T0 to T25 following the introduction into the rumen of S35 - labelled sodium sulphate.

		Distance (cms) from origin														
Sample	Time 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sheep 1.																
T0	35	27	30	25	42	38	37	28	29	31	33	24	26	21	18	23
T5	37	36	908	143	4966	115	109	433	103	76	56	45	48	36	42	33
T10	32	41	1055	167	987	103	132	1034	95	82	64	52	64	51	49	38
T15	41	35	826	132	542	121	127	742	72	67	58	49	**46**	**55**	**38**	41
T20	33	28	529	141	326	113	116	527	68	58	35	62	35	41	35	45
T25	43	34	272	87	105	67	76	155	67	43	28	37	31	35	37	36
Sheep 2.																
T0	28	31	35	26	25	28	29	28	30	32	30	33	38	42	31	29
T5	35	38	267	184	2791	276	235	1421	96	85	56	48	39	35	39	37
T10	38	42	794	142	435	241	228	674	104	93	49	39	34	29	35	26
T15	32	34	453	109	398	183	146	501	93	76	63	51	28	29	25	21
T20	29	37	320	115	205	174	139	328	97	**89**	**52**	**42**	**33**	**25**	29	25
T25	34	41	177	42	76	87	58	104	63	59	45	40	26	28	31	27

Appendix 1. Experiment A. (cont.)

Sample Time	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sheep 3.																
T0	36	32	29	35	28	36	41	39	37	36	27	26	33	28	34	32
T5	39	43	141	76	5837	207	105	173	105	72	45	24	34	25	37	30
T10	42	35	461	121	529	210	127	844	114	75	49	31	28	26	31	25
T15	31	28	347	142	307	173	119	600	87	69	56	22	35	28	32	26
T20	34	33	270	122	204	115	89	326	64	74	38	29	29	31	27	21
T25	27	29	171	87	126	84	75	149	75	80	43	20	23	20	23	18

Appendix 2. Experiment B(1)

Analysis of variance of the effect of anions other than group V1 anions on inorganic sulphate reduction in the rumen.

Due to	df	S.S.	M.S.	F.
Treatment	2	0.07	0.035	0.7 N.S.
Time	10	2.57	0.26	5.2 *
Error	20	1.16	0.05	
Total	32	3.8		

Analysis of variance of concentration of steam volatile fatty acids in the rumen of sheep fed group V1 anions.

Due to	df	S.S.	M.S.	F.
Treatment	5	3.5	0.7	0.6 N.S.
Time	5	428	85.6	71.3 ***
Error	25	29.5	1.2	
Total	35	461		

N.S. Not significant; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Appendix 3. Experiment B (2)

Rumen sulphide levels (μg $S^{=}$ / ml rumen fluid) of sheep fed four levels of dietary molybdenum (means of 4 sheep).

		Dietary	Molybdenum	Level	(ppm).
Sample Time	<0.7	40	240	720	1440
0	4.0	4.5	1.4	1.6	1.2
30	5.3	6.8	1.3	1.5	1.1
90	5.5	7.5	1.5	1.6	0.9
150	4.8	7.9	1.4	1.1	0.7
210	4.9	8.3	1.4	1.1	0.6
270	5.2	9.2	1.6	0.8	0.5
330	5.7	9.0	1.4	0.9	0.6
390	6.4	10.4	1.6	1.0	0.7
450	5.1	10.3	1.7	1.1	0.6
510	5.2	10.1	2.1	1.0	0.6

Analysis of variance.

Due to	df	S.S.	M.S.	F.	Prob.
Sheep	3	946.15	315.38	2.28	0.82
Treatments	3	15888.57	5296.19	38.36	0.99
Times of Treatments	3	750.65	250.22	1.81	0.75
Error	6	828.49	138.08		
Total	15	18413.86			

F significant at 5% if Prob > 0.95.

Appendix 3. Experiment B(2)

Rumen molybdenum levels (ppm) of sheep fed four levels of dietary molybdenum (means of 4 sheep).

	Dietary Molybdenum Levels (ppm).			
Sample Time	40	240	720	1440
0	2.5	11.0	52.0	96.0
30	3.5	11.0	50.0	99.0
90	2.0	12.0	51.0	95.0
150	3.0	11.0	48.0	89.0
210	2.5	11.0	49.0	92.0
270	3.0	11.0	46.5	95.5
330	3.0	10.5	42.0	91.5
390	2.0	11.0	50.0	104.5
450	2.5	11.5	47.0	95.5
510	3.0	11.5	47.0	97.5

Analysis of variance.

Due to	df	S.S.	M.S.	F.	Prob.
Sheep	3	141067.92	47022.64	2.22	0.81
Treatments	3	2146462.05	715487.35	33.85	0.99
Times of Treatments	3	80199.42	26733.14	1.26	0.63
Error	6	126811.47	21135.24		
Total	15				

F. significant at 5% if Prob $>$ 0.95.

Appendix 3. Experiment B(2)

Rumen PH levels of sheep fed four levels of dietary molybdenum (means of 4 sheep).

Sample Time	Dietary Molybdenum Level (ppm).				
	<0.7	40	240	720	1440
0	7.1	7.2	7.0	7.1	6.9
30	7.0	7.1	6.9	7.0	6.8
90	6.9	7.0	6.8	6.8	6.7
150	6.7	6.9	6.7	6.7	6.7
210	6.7	6.7	6.6	6.6	6.6
270	6.6	6.7	6.6	6.7	6.5
330	6.7	6.7	6.6	6.7	6.5
390	6.6	6.7	6.7	6.6	6.6
450	6.6	6.6	6.5	6.6	6.6
510	6.7	6.5	6.6	6.6	6.5

Analysis of variance.

Due to	df	S.S.	M.S.	F.	Prob.
Sheep	3	51.33	17.11	8.98	0.99
Treatments	3	5.58	1.86	0.98	0.54
Times of Treatments	3	119.79	39.93	20.96	0.99
Error	6	11.43	1.90		
Total	15	188.12			

F significant at 5% level if Prob > 0.95

Appendix 3. Experiment B(2)

Rumen redox potentials of sheep fed four levels of dietary molybdenum (means of 4 sheep).

Sample Time	Dietary Molybdenum Level (ppm).				
	<0.7	40	240	720	1440
0	-330	-330	-325	-320	-290
30	-330	-330	-325	-320	-295
90	-320	-345	-315	-310	-285
150	-300	-330	-310	-300	-280
210	-295	-320	-310	-295	-290
270	-300	-315	-300	-300	-285
330	-300	-310	-300	-295	-280
390	-305	-315	-305	-300	-280
450	-305	-315	-305	-305	-290
510	-305	-320	-305	-305	-295

Analysis of variance.

Due to	df	S.S.	M.S.	F	Prob.
Sheep	3	99631.25	33210.42	0.92	0.51
Treatments	3	278818.75	92939.58	2.57	0.85
Times of Treatments	3	149731.25	49910.42	1.38	0.66
Error	6	216712.5	36118.75		
Total	15	744893.75			

F significant at 5% level if Prob > 0.95

Appendix 3. Experiment B(2)

Rumen volatile fatty acid concentrations of sheep fed four levels of dietary molybdenum (means of 4 sheep).

Sample Time	Dietary Molybdenum Level (ppm).				
	< 0.7	40	240	720	1440
0	55.0	57.0	56.5	52.5	54.5
30	52.0	56.5	58.0	53.0	56.5
90	55.5	61.5	60.0	58.0	57.0
150	61.0	62.5	61.5	57.0	57.5
210	64.0	65.5	59.0	60.5	60.5
270	63.5	63.5	64.0	56.5	65.0
330	66.5	67.5	62.5	57.5	63.0
390	64.5	67.0	62.0	60.0	61.0
450	62.5	69.0	66.0	58.5	63.0
510	61.0	70.5	61.5	60.0	64.5

Analysis of variance.

Due to	df	S.S.	M.S.	F.	Prob.
Sheep	3	16597.42	5532.47	1.87	0.76
Treatments	3	3540.55	1180.18	0.40	0.24
Times of Treatments	3	22880.67	7626.89	2.58	0.85
Error	6	17741.72	2956.95		
Total	15	60760.36			

F significant at 5% level if Prob > 0.95

Appendix 3. Experiment B(2)

Analysis of variance of cotton thread digestion in sheep fed four levels of dietary molybdenum (means of 4 sheep).

Due to	df	S.S.	M.S.	F
Sheep	3	22.19	7.4	1.8 N.S.
Treatment	3	22.19	7.4	1.8 N.S.
Error	9	36.56	4.06	
Total	15	80.94		

Analysis of variance of total bacteria numbers in sheep fed four levels of dietary molybdenum (means of 4 sheep).

Due to	df	S.S.	M.S.	F
Sheep	3	3700.4	1233.5	2.1 N.S.
Treatment	3	2535.6	845.2	1.4 N.S.
Error	9	5283.6	587.1	
Total	15	11519.6		

Analysis of variance of sulphate - reducing bacteria numbers in sheep fed four levels of dietary molybdenum (means of 4 sheep).

Due to	df	S.S.	M.S.	F
Sheep	3	2633192.8	877730.9	3.75 N.S.
Treatment	3	551179.23	183726.4	0.8 N.S.
Error	9	2103496.7	233721.8	
Total	15	5287868.7		

N.S. = Not Significant

Appendix 3. Experiment B(2)

Rumen sulphate levels (μg $SO_4^{=}$ /ml rumen fluid) of sheep fed four levels of dietary molybdenum (means of 4 sheep).

Sample Time	Dietary Molybdenum Level (ppm).				
	<0.7	40	240	720	1440
0	116	49	43	68	95
30	258	112	144	161	183
90	155	99	120	132	140
150	150	93	107	155	129
210	128	72	103	118	135
270	118	79	92	137	136
330	117	63	86	128	138
390	117	70	99	104	130
450	104	63	77	122	148
510	99	56	49	120	136

Analysis of variance.

Due to	df	S.S.	M.S.	F	Prob.
Sheep	3	580537.69	193512.56	3.5	0.91
Treatments	3	962366.19	320788.73	5.8	0.96
Times of Treatments	3	1275670.69	425223.56	7.69	0.98
Error	6	331605.87	55267.64		
Total	15	3150180.44			

F significant at 5% if Prob > 0.95

Appendix 4. Experiment B (3)

Rumen sulphide levels ($\mu g\ S^{=}$ / ml rumen fluid) of sheep fed diets without sulphate with no molybdenum, without sulphate with added molybdenum, sulphate with no molybdenum and sulphate with added molybdenum (means of 4 sheep).

	Dietary Supplements			
Sample Time	-S-Mo	-S+Mo	+S-Mo	+S+Mo
0	0	0	1.8	1.4
30	0	0	3.3	1.3
90	0	0	3.8	1.3
150	0	0	3.4	1.3
210	0	0	3.6	1.5
270	0	0	4.0	1.0
330	0	0	4.4	1.6
390	0	0	5.3	1.9
450	0	0	5.5	2.2
510	0	0	5.1	1.7

Analysis of variance.

Due to	df	S.S.	M.S.	F	Prob.
Sheep	3	842.86	280.95	0.81	0.46
Treatments	3	4266.37	1422.12	4.09	0.93
Times of Treatments	3	1226.03	408.68	1.18	0.60
Error	6	2085.69	347.61		
Total	15	8420.94			

F significant at 5% if Prob $\geq$ 0.95

Appendix 4. Experiment B(3)

Rumen molybdenum levels (ppm) of sheep fed diets without sulphate with no molybdenum, without sulphate with added molybdenum, sulphate with no molybdenum and sulphate with added molybdenum. (Means of four sheep).

	Dietary Supplements			
Sample Time	-S-Mo	-S+Mo	+S-Mo	+S+Mo
0	0	37	0	49
30	0	55	0	66
90	0	54	0	74
150	0	58	0	59
210	0	61	0	58
270	0	50	0	62
330	0	48	0	61
390	0	54	0	59
450	0	55	0	58
510	0	53	0	62

Analysis of variance.

Due to	df	S.S.	M.S.	F	Prob.
Sheep	3	31190.5	10396.83	0.49	0.29
Treatments	3	1288419.0	429473.0	20.14	0.99
Time of Treatments	3	24801.5	8267.17	0.39	0.23
Error	6	127920.0	21320.0		
Total	15	1472331.0			

F significant at 5% level if Prob $\geq$ 0.95

Appendix 4. Experiment B(3)

Rumen PH in sheep fed diets without sulphate with no molybdenum, without sulphate with added molybdenum, sulphate with no molybdenum and sulphate with added molybdenum (means of 4 sheep).

	Dietary Supplements			
Sample Time	-S-Mo	-S+Mo	+S-Mo	+S+Mo
0	7.3	7.5	7.5	7.5
30	7.3	7.4	7.4	7.3
90	7.1	7.3	7.1	7.0
150	6.9	7.2	6.8	6.9
210	6.8	7.1	6.8	6.9
270	6.9	7.1	6.8	6.9
330	6.8	7.0	6.9	6.9
390	6.8	7.0	7.0	7.0
450	6.8	7.0	7.0	7.1
510	6.9	7.1	7.2	7.2

Analysis of variance.

Due to	df	S.S.	M.S.	F	Prob.
Sheep	3	12.38	4.13	2.59	0.85
Treatments	3	7.82	2.61	1.64	0.72
Times of Treatments	3	6.16	2.05	1.29	0.64
Error	6	9.56	1.59		
Total	15	35.93			

F significant at 5% level if Prob $>$ 0.95.

Appendix 4. Experiment B(3)

Rumen redox potentials of sheep fed diets without sulphate with no molybdenum, without sulphate with added molybdenum, sulphate with no molybdenum and sulphate with added molybdenum. (means of 4 sheep).

	Dietary Supplements.			
Sample Time	-S-Mo	-S+Mo	+S-Mo	+S+Mo
0	-390	-400	-390	-400
30	-380	-380	-380	-370
90	-360	-370	-350	-360
150	-340	-350	-350	-350
210	-350	-350	-340	-350
270	-350	-360	-360	-350
330	-360	-360	-370	-360
390	-370	-370	-380	-380
450	-390	-380	-390	-390
510	-390	-390	-390	-390

Analysis of variance

Due to	df	S.S.	M.S.	F	Prob.
Sheep	3	60118.75	20039.58	1.55	0.70
Treatments	3	5868.75	1956.25	0.15	0.07
Times of Treatments	3	2868.75	956.25	0.07	0.03
Error	6	77537.5	12922.92		
Total	15	146393.75			

F significant at 5% level if Prob $>$ 0.95

Appendix 4. Experiment B(3)

Rumen volatile fatty acid concentrations (mMoles/litre rumen fluid) of sheep fed diets without sulphate with no molybdenum, without sulphate with added molybdenum, sulphate with no molybdenum and sulphate with added molybdenum (means of 4 sheep).

	Dietary Supplements			
Sample Time	-S-Mo	-S+Mo	+S-Mo	+S+Mo
0	43.0	38.0	36.0	43.0
30	45.5	41.5	45.0	46.5
90	53.5	44.5	57.0	56.0
150	58.0	46.0	64.0	62.5
210	58.0	49.5	65.5	63.5
270	58.0	51.5	67.0	62.5
330	60.0	51.5	61.5	61.5
390	60.0	55.5	62.0	58.0
450	62.5	58.5	63.5	58.0
510	60.5	56.0	61.0	56.6

Analysis of variance.

Due to	df	S.S.	M.S.	F	Prob.
Sheep	3	47091.92	15697.31	3.61	0.91
Treatments	3	19118.17	6372.72	1.47	0.68
Times of Treatments	3	14919.30	4973.09	1.14	0.59
Error	6	26095.22	4349.20		
Total	15	107224.61			

F significant at 5% level if Prob $>$ 0.95

Appendix 4. Experiment B(3)

Analysis of variance of cotton thread digestion of sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Due to	df	S.S.	M.S.	F
Sheep	3	0.5	0.17	0.01 N.S.
Treatments	3	3251.5	1083.8	67.7 ***
Error	9	144	16	
Total	15	3396		

Analysis of variance of total bacteria numbers of sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Due to	df	S.S.	M.S.	F
Sheep	3	108.3	36.1	0.22 N.S.
Treatments	3	639.02	213	1.3 N.S.
Error	9	1471.2	163.5	
Total	15	2218.5		

Analysis of variance of sulphate - reducing bacteria numbers of sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Due to	df	S.S.	M.S.	F
Sheep	3	48969.6	16323.2	1. N.S.
Treatments	3	3777270	125909.0	78.09 ***
Error	9	145108.7	16123.2	
Total	15	3971348.3		

*** = $P < 0.001$; N.S. = Not Significant.

Appendix 4. Experiment B(3)

Rumen sulphate levels (μg $SO_4^=$ /ml rumen fluid) of sheep fed diets without sulphate with no molybdenum without sulphate with added molybdenum sulphate with no molybdenum and sulphate with added molybdenum (means of 4 sheep).

	Dietary Supplements			
Sample Time	-S-Mo	-S+Mo	+S-Mo	+S+Mo
0	3	2	48	45
30	5	4	144	135
90	6	4	144	139
150	6	7	141	129
210	5	7	129	108
270	4	6	115	114
330	4	6	104	103
390	4	8	96	97
450	3	7	83	66
510	2	7	80	84

Analysis of variance

Due to	df	S.S.	M.S.	F	Prob.
Sheep	3	1048590.69	349530.23	2.45	0.84
Treatment	3	3128616.19	1042872.06	7.31	0.98
Times of Treatment	3	549184.69	183061.56	1.28	0.64
Error	6	855410.87	142568.48		
Total	15	5581802.44			

F significant at 5% level if Prob $>$ 0.95

Appendix 4. Experiment B(3)

Analysis of variance of molybdenum balances in sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Molybdenum Balance Figures			
-S-Mo	-S+Mo	+S-Mo	+S+Mo
0	0.61	0	0.59
0	0.72	0	0.44
0	0.15	0	0.04
0	0.23	0	-0.03

Analysis of variance

Due to	df	S.S.	M.S.	F
Sheep	3	0.25	0.08	2.7 N.S.
Treatments	3	0.53	0.18	6 *
Error	9	0.25	0.03	
Total	15	1.04		

* = $P < 0.05$; N.S. = Not Significant

Appendix 4. Experiment B(3)

Analysis of variance of nitrogen balances in sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Nitrogen Balance Figures			
-S-Mo	-S+Mo	+S-Mo	+S+Mo
3.86	1.03	2.02	2.01
3.98	1.70	2.08	1.36
2.43	-1.71	2.43	2.97
2.27	0.31	1.98	1.59

Analysis of variance

Due to	df	S.S.	M.S.	F
Sheep	3	2.08	0.7	2.3 N.S.
Treatment	3	16.16	5.4	18 ***
Error	9	2.74	0.3	
Total	15	20.98		

*** = $P < 0.001$; N.S. = Not Significant

Appendix 4. Experiment B(3)

Analysis of variance of sulphur balances in sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Sulphur Balance Figures			
-S-Mo	-S+Mo	+S-Mo	+S+Mo
-0.04	0.003	0.03	0.02
0.02	0.02	0.02	0.06
0.05	0.02	0.07	0.02
0.02	0.01	0.03	0.01

Analysis of variance

Due to	df	S.S.	M.S.	F
Sheep	3	0.002	0.0007	2.3 N.S.
Treatments	3	0.002	0.0007	2.3 N.S.
Error	9	0.003	0.0003	
Total	15	0.007		

* = $P < 0.05$; N.S. = Not Significant

Appendix 4. Experiment B(3)

Analysis of variance of feed intakes in sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Due to	df	S.S.	M.S.	F
Sheep	3	84517.7	28172.6	3.99 *
Treatment	3	37233.6	12411.2	1.76 N.S.
Error	9	63446.6	7049.6	
Total	15	185197.9		

Analysis of variance of urine volumes in sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Due to	df	S.S.	M.S.	F.
Sheep	3	390532.2	130177.4	1.5 N.S.
Treatment	3	424225.2	141408.4	1.63 N.S.
Error	9	781246.3	86805.2	
Total	15	1596003.8		

Analysis of variance of faeces weights in sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Due to	df	S.S.	M.S.	F
Sheep	3	2808.5	936.2	0.64 N.S.
Treatments	3	11315.5	3771.8	2.58 N.S.
Error	9	13134.0	1459.3	
Total	15	27258.0		

* = $P < 0.05$; N.S. = Not Significant

Appendix 4. Experiment B(3)

Feed intake (dry matter), urine volume and faecal weights (dry matter) in sheep fed diets without added sulphate and added sulphate with and without added molybdenum (means of four sheep).

Treatment	Feed Intake (g)	Urine Vol.(ml.)	Faeces Wgt.(g)
-S-Mo	510.2	403.2	236.5
-S+Mo	449.2	792.0	173.7
+S-Mo	585.2	418.5	241.0
+S+Mo	521.0	427.7	218.7

Appendix 5. Experiment 8(4)

Rumen sulphide levels ($\mu g\ S^{=}$ /ml rumen fluid) of sheep fed six levels of dietary molybdenum (means of six sheep).

Sample Time	Dietary Molybdenum Level (ppm).						
	0.7	20	48	76	104	132	160
0	3.8	4.3	4.2	2.9	2.5	1.8	1.4
30	4.3	4.8	6.6	3.5	3.3	2.4	1.5
90	4.3	5.9	7.3	4.3	3.4	2.0	1.5
150	5.2	5.8	7.8	4.5	3.8	1.6	1.5
210	5.3	6.7	8.1	4.5	3.9	1.7	1.5
270	6.7	7.2	8.0	4.8	4.4	1.7	1.5
330	6.2	7.2	8.2	5.2	3.8	1.5	1.4
390	6.1	7.5	9.1	4.9	3.6	1.5	1.3
450	6.3	6.9	8.6	4.8	3.8	1.4	1.3
510	6.4	6.7	7.6	4.7	3.2	1.4	1.2

Analysis of variance

Due to	df	S.S.	M.S.	F	Prob.
Sheep	5	2939.68	587.94	1.52	0.77
Treatments	5	17996.16	3599.23	9.30	0.99
Times of Treatment	5	1881.33	376.27	0.97	0.54
Error	20	7741.79	387.09		
Total	35	30558.97			

F Significant at 5% level if Prob $>$ 0.95

Appendix 5. Experiment 8(4)

Rumen sulphate levels ($\mu g SO_4^{=}$/ml rumen fluid) in sheep fed six levels of dietary molybdenum (means of six sheep).

Sample Time (0	7	20	Dietary Molybdenum Levels(ppm). 48	76	104	132	160
0	75	40	19	60	30	36	23
30	175	146	135	120	113	142	155
90	161	144	139	137	146	153	150
150	150	147	137	114	135	152	152
210	125	109	110	122	119	125	129
270	119	102	112	115	123	110	113
330	112	91	102	95	123	115	107
390	110	87	81	97	99	91	89
450	93	83	63	82	92	96	87
510	89	67	60	73	94	96	78

Analysis of variance

Due to	df	S.S.	M.S.	F	Prob.
Sheep	5	289942.47	57988888.49	0.88	0.49
Treatments	5	262262.47	52452.49	0.80	0.43
Times of Treatments	5	5320443.81	1064088.76	16.22	1.00
Error	20	1311828.89	65591.44		
Total	35	7184477.64			

F significant at 5% level if Prob $>$ 0.95

Appendix 5. Experiment 8(4)

Rumen molybdenum levels (ppm) in sheep fed six levels of dietary molybdenum (means of six sheep).

Sample Time		Dietary Molybdenum Levels (ppm).					
	0.7	20	48	76	104	132	160
0	0	2.5	3.0	5.0	5.5	8.0	8.5
30	0	3.0	4.5	7.0	8.5	11.5	14.0
90	0	3.0	4.5	7.5	8.0	11.0	12.0
150	0	2.5	4.5	7.5	8.0	11.0	12.0
210	0	1.5	3.5	5.0	7.5	9.0	10.5
270	0	3.0	3.0	5.5	8.5	9.5	10.0
330	0	2.5	2.5	5.5	8.0	9.0	11.0
390	0	2.0	2.5	5.5	7.5	9.5	10.5
450	0	2.5	3.0	5.5	8.0	8.5	9.0
510	0	2.0	2.5	6.0	8.0	8.0	9.5

Analysis of variance

Due to	df	df	S.S.	M.S.	F	Prob.
Sheep		5	1720.17	344.03	1.73	0.82
Treatments		5	28863.08	5772.62	28.96	1.00
Times of Treatments		5	29511.25	5902.25	29.61	1.00
Error		20	3986.25	199.31		
Total		35	64080.75			

F significant at 5% level if Prob $>$ 0.95

Appendix 5. Experiment B(4)

Rumen pH levels in sheep fed six levels of dietary molybdenum (means of six sheep).

Sample Time	Dietary Molybdenum Levels (ppm)						
	0.7	20	48	76	104	132	160
0	7.3	7.4	7.5	7.4	7.4	7.5	7.5
30	7.2	7.3	7.4	7.3	7.3	7.4	7.4
90	6.9	7.1	7.1	7.1	7.2	7.1	7.1
150	6.8	6.8	6.8	6.9	6.9	6.9	6.8
210	6.8	6.7	6.7	6.8	6.8	6.8	6.8
270	6.8	6.7	6.7	6.8	6.8	6.8	6.8
330	6.8	6.7	6.8	6.8	6.8	6.8	6.8
390	6.8	6.8	6.8	6.8	6.9	6.9	6.9
450	6.8	6.9	6.8	6.8	6.8	6.9	6.8
510	6.9	6.9	6.9	6.9	6.9	7.0	7.0

Analysis of variance

Due to	df	S.S	M.S.	F.	Prob.
Sheep	5	40.95	8.19	13.7	1.00
Treatment	5	1.87	0.37	0.63	0.32
Time of Treatment	5	65.45	13.09	21.92	1.00
Error	20	11.94	0.60		
Total	35	120.21			

F significant at 5% level is Prob > 0.95.

Appendix 5. Experiment B(4)

Rumen redox potentials in sheep fed six levels of dietary molybdenum (means of six sheep).

Sample Time	Dietary Molybdenum Levels (ppm).						
	<0.7	20	48	76	104	132	160
0	-370	-390	-400	-390	-400	-390	-390
30	-360	-380	-380	-380	-380	-380	-380
90	-350	-360	-360	-360	-370	-350	-360
150	-350	-350	-350	-350	-350	-340	-350
210	-350	-350	-350	-340	-350	-340	-340
270	-360	-350	-350	-340	-350	-350	-350
330	-360	-360	-360	-360	-360	-360	-360
390	-360	-370	-370	-370	-370	-370	-380
450	-360	-390	-390	-380	-390	-390	-390
510	-360	-390	-390	-390	-390	-390	-390

Analysis of variance

Due to	df	S.S.	M.S.	F	Prob.
Sheep	5	30188.89	6037.78	2.91	0.96
Treatments	5	18188.89	3637.78	1.75	0.83
Times of Treatments	5	11622.22	2324.44	1.12	0.62
Error	20	41522.22	2076.11		
Total	35	101522.22			

F significant at 5% level if Prob $>$ 0.95

Appendix 5. Experiment B(4)

Rumen volatile fatty acids (m Moles/litre rumen fluid) in sheep fed six levels of dietary Molybdenum (means of six sheep).

	Dietary Molybdenum Levels (ppm).						
Sample Time	0.7	20	48	76	104	132	160
0	46.5	40.5	44.0	43.0	42.5	41.5	39.5
30	49.5	41.0	45.5	45.5	43.5	44.5	40.0
90	59.5	56.0	56.5	54.5	50.5	52.5	52.5
150	58.0	63.5	65.0	58.5	56.0	59.5	59.5
210	63.0	61.5	67.5	60.0	59.0	61.5	62.5
270	62.0	60.0	65.0	61.0	58.5	61.5	61.5
330	60.0	61.0	65.0	61.0	62.0	59.0	59.0
390	58.5	58.5	64.0	60.5	59.5	58.5	58.5
450	63.0	59.0	64.0	60.0	60.5	59.5	61.5
510	58.5	57.0	62.5	57.5	57.5	56.5	57.0

Analysis of variance

Due to	df	S.S.	M.S.	F	Prob.
Sheep	5	42313.28	8462.66	4.90	0.99
Treatments	5	10649.28	2129.86	1.23	0.67
Times of Treatments	5	58338.78	11667.76	6.76	0.99
Error	20	34525.89	1726.29		
Total	35	145827.23			

F significant at 5% level if Prob $>$ 0.95

Appendix 5. Experiment B(4)

Analysis of variance of cotton thread digestion in sheep fed six levels of dietary molybdenum (means of six sheep).

Due to	df	S.S.	M.S.	F
Sheep	5	7.8	1.56	1.18 N.S.
Treatments	5	13.1	2.62	1.98 N.S.
Error	25	33.1	1.32	
Total	35	54		

Analysis of variance of total bacteria numbers in sheep fed six levels of dietary molybdenum (means of six sheep).

Due to	df	S.S.	M.S.	F
Sheep	5	416.6	83.32	1.05 N.S.
Treatments	5	84.6	16.9	0.21 N.S.
Error	25	1978.6	79.15	
Total	35	2479.8		

Analysis of variance of sulphate - reducing bacteria numbers in sheep fed six levels of dietary molybdenum (means of six sheep).

Due to	df	S.S.	M.S.	F
Sheep	5	118653.4	23730.7	0.96 N.S.
Treatments	5	107690.6	21538.1	0.87 N.S.
Error	25	618420.5	24736.8	
Total	35	844764.5		

N.S. = Not Significant

Appendix 5. Experiment B(4)

Analysis of variance of feed intakes in sheep fed six levels of dietary molybdenum (means of six sheep).

Due to	df	S.S.	M.S.	F
Sheep	5	25167.87	5033.6	1.38 N.S.
Treatments	5	14656.2	2931.2	0.8 N.S.
Error	25	91112.9	3644.5	
Total	35	130936.9		

Analysis of variance of urine volumes in sheep fed six levels of dietary molybdenum (means of six sheep).

Due to	df	S.S.	M.S.	F.
Sheep	5	141802	28360	1.00 N.S.
Treatments	5	16091	3218	0.10 N.S.
Error	25	706318	28252	
Total	35	864211		

Analysis of variance of faeces weights in sheep fed six levels of dietary molybdenum (means of six sheep).

Due to	df	S.S.	M.S.	F
Sheep	5	13958	2792	2.4 N.S.
Treatments	5	899	180	0.15 N.S.
Error	25	28957	1158	
Total	35	43814		

N.S. = Not Significant

Appendix 5. Experiment B(4)

Analysis of variance of nitrogen balance figures in sheep fed six levels of dietary molybdenum (means of six sheep).

Dietary Molybdenum Levels (ppm).					
20	48	76	104	132	160
0.45	0.37	0.36	0.37	0.24	0.26
0.26	0.25	0.26	0.25	0.11	0.19
0.15	0.33	0.12	0.21	0.21	0.35
0.31	0.17	0.26	0.15	0.20	0.33
0.28	0.15	0.17	0.27	0.16	0.23
0.37	0.25	0.32	0.21	0.27	0.26

Analysis of Variance

Due to	df	S.S.	M.S.	F
Sheep	5	0.07	0.014	2.8 *
Treatments	5	0.03	0.006	1.2 N.S.
Error	25	0.13	0.005	
Total	35	0.23		

* = $P < 0.05$; N.S. = Not Significant

Appendix 5 Experiment B(4)

Analysis of variance of sulphur balance figures in sheep fed six levels of dietary molybdenum (means of six sheep).

Dietary Molybdenum Levels (ppm).

20	48	76	104	132	160
0.01	0.03	0.02	0.01	0.03	-0.02
0.02	0.01	0.02	0.03	0.02	0.01
-0.01	0.02	0.01	-0.02	-0.03	0.02
0.03	0.01	0.02	0.03	0.02	0.02
0.02	0.03	0.01	0.02	0.01	0.06
0.03	0.02	0.02	0.03	0.03	0.03

Analysis of Variance

Due to	df	S.S.	M.S.	F	
Sheep	5	0.03	0.006	6.0	**
Treatments	5	0.002	0.0004	0.4	N.S.
Error	25	0.027	0.001		
Total	35	0.062			

** = $P < 0.001$; N.S. = Not Significant

Appendix 5. Experiment B (4)

Feed intake (dry matter), urine volumes and faecal weights (dry matter) in sheep fed six levels of dietary molybdenum (means of six sheep).

Dietary Mo Levels (ppm)	Feed Intake (g)	Urine Vol.(ml)	Faeces Wgt(g)
20	595.3	387.6	230.0
48	606.8	412.8	234.0
76	611.3	412.5	243.0
104	580	415.2	235.0
132	565.0	380.6	227.0
160	557.6	357.7	235.7

Appendix 5. Experiment B (4)

Analysis of variance of molybdenum balance figures in sheep fed six levels of dietary molybdenum (means of six sheep).

Dietary Molybdenum Levels (ppm)					
20	48	76	104	132	160
0.15	0.21	0.66	0.73	0.70	0.60
0.09	0.08	0.16	0.04	0.19	0.17
0.53	0.59	0.11	0.22	0.29	0.60
-0.03	0.01	0.54	0.77	0.78	0.13
0.17	0.41	0.66	0.66	0.76	0.17
0.24	0.14	0.62	0.66	0.72	0.58

Analysis of Variance

Due to	df	S.S.	M.S.	F
Sheep	5	0.65	0.13	2.6 *
Treatments	5	0.7	0.14	2.8 *
Error	25	1.21	0.05	
Total	35	2.56		

* = $P < 0.05$.